UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

Impact of metabolic resources and infiltrating immune cells on protective responses against solid tumors

Neidy Varela Rodrigues

Orientador:
Professor Doutor Sergio Jerónimo Rodrigues Dias

Co-orientador:
Professor Doutor Bruno Silva-Santos

Tese especialmente elaborada para obtenção do grau do Doutor em Ciências Biomédicas, especialidade de Biologia Celular e Molecular

2019

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

Impact of metabolic resources and infiltrating immune cells on protective responses against solid tumors

Neidy Varela Rodrigues

Orientador:
Professor Doutor Sergio Jerónimo Rodrigues Dias

Co-orientador:
Professor Doutor Bruno Silva-Santos

Tese especialmente elaborada para obtenção do grau do Doutor em Ciências Biomédicas, especialidade de Biologia Celular e Molecular

Júri:

Presidente:
- Doutor João Eurico Cortez Cabral da Fonseca, Professor Catedrático e Vice-Presidente do concelho científico da Faculdade de Medicina da Universidade de Lisboa

Vocais:
- Doutora Joana Cancela de Amorim Falcão Paredes, Professora Afiliada da Faculdade de Medicina do Porto;
- Doutora Maria José Cardoso Oliveira, Investigadora Principal do Instituto Nacional de Engenharia Biomédica da Universidade do Porto;

- Doutor Luís Ricardo Simões da Silva Graça, Professor Associado com Agregação da Faculdade de Medicina da Universidade de Lisboa;

- Doutor João Pedro Taborda Barata, Professor Associado Convidado da Faculdade de Medicina da Universidade de Lisboa;

- Doutor Sérgio Jerónimo Rodrigues Dias, Professor Associado Convidado da Faculdade de Medicina da Universidade de Lisboa (Orientador).

2019

As opiniões expressas nesta publicação são da exclusiva responsabilidade do seu autor.

Supervisors:

Prof. Dr. Sérgio Jerónimo Rodrigues Dias, P.h.D

Prof. Dr. Bruno Silva-Santos, P.h.D

Instituto de Medicina Molecular-João Lobo Antunes

Faculdade de Medicina,

Universidade de Lisboa.

A presente dissertação foi realizada nos laboratórios dirigidos por Sérgio Dias e Bruno Silva Santos no Instituto de Medicina Molecular João Lobo Antunes, e foi apresentada à Faculdade de Medicina da Universidade de Lisboa, para obtenção do grau de Doutor em Ciências Biomédicas.

Todas as afirmações efetuadas no presente documento são da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à Faculdade de Medicina de Lisboa pelos conteúdos nele apresentados.

A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 27 de Novembro de 2019.

O trabalho aqui apresentado foi cofinanciado
Pelo o Programa de Pós-Graduação Ciência para o Desenvolvimento (PGCD), Fundação para a Ciência e Tecnologia: (PTDC/DTP-PIC/4931/2014 para B.S.-S, Fundo iMM-LAÇO (para S.D.) e bolsa de
Doutoramento SFRH/BD/ 113756 /2015 para N.V.R

Para os meus pais,

Henrique e Ângela

E ao meu filho Angelo

"Education is the most powerful weapon which you can use to change the world"

Nelson Mandela

TABLE OF CONTENTS

ACKNOWLEDGEMENTS / AGRADECIMENTOS xvii

ABBREVIATIONS xix

Sumário xiii

Summary xxv

1-GENERAL INTRODUCTION **1**

1.1Hallmarks of cancer 1

1.1.2 The tumor immunosurveillance hypothesis/theory 2

1.1.3 Tumor infiltrating lymphocytes 3

1.2 Gamma delta (γδ) T cells 3

1.2.1. Vγ9Vδ2 T cells in immunity 5

1.2.2. Recognition of non-peptide antigens by human Vγ9Vδ2 T cells 8

1.2.3. TCR-mediated activation 10

1.2.4. Cytotoxic receptors 12

1.2.4.1. NGK2D 12

1.2.4.2 DNAM-1 (CD226) 12

1.2.5. V9γVδ2 T cells in cancer immunotherapy 13

1.3- Metabolism and immune cell function. 15

1.3.1 Cellular cholesterol: physiological and homeostasis 16

1.3.2 Cholesterol in immune cells 19

1.4- Systemic cholesterol and tumor progression 21

1.5. Objectives of thesis 23

2-LDL-cholesterol uptake inhibits the activation and anti-tumor functions of human Vγ9Vδ2 T cells 24

2.1- Abstract 24
2.2 Introduction 24
2.3 Material and methods 27
2.3.1 Cell culture and in vitro killing assays 27
2.3.2 Flow cytometry analysis 28
2.3.3 Quantitative Real-Time PCR 29
2.3.4 Histological analysis 30
2.3.5 In vivo tumour experiments 30
2.3.6 Statistical analysis 31
2.4 Results 32
2.4.1 Activated Vγ9Vδ2 T cells uptake LDL-cholesterol via LDL-Receptor 32
2.4.2 LDL-cholesterol inhibits Vγ9Vδ2 T cell activation and cytokine production 35
2.4.3 LDL-cholesterol downregulates NKG2D and DNAM-1 and reduces γδ T cell cytotoxicity in vitro 38
2.4.4 LDL-cholesterol limits the anti-tumour therapeutic effect of human γδ T cells in vivo 40
2.5 Discussion 44
2.6 References 46
3. Conclusion and Future perspectives 53
4. References 59

Figure índex

Chapter 1

GENERAL INTRODUCTION

FIGURE 1: Ligands recognized by the γδ t cell antigen receptor. 7

FIGURE 2: Mevalonate and mep (2-c-methyl-d-erythritol 4-phosphate) pathways for isoprenoid biosynthesis. 9

FIGURE 3: T cell receptor signaling cascade. 11

FIGURE 4: Strategies in vivo: administration of compounds that activate γδ t cells or adoptive transfer of ex vivo expanded γδ t cells. 14

FIGURE 5: Cholesterol as signaling molecule in t cells. 21

FIGURE 6: Lipid metabolism and cancer progression 22

FIGURE 7: Schematic of the approach and conclusions of this thesis. 54

FIGURE 8: Future plans 58

Chapter 2

LDL-cholesterol uptake inhibits the activation and anti-tumor functions of human Vγ9Vδ2 T cell

FIGURE 2.4.1: Activated Vγ9Vδ2 T cells uptake LDL-cholesterol via LDL-Receptor 33

FIGURE 2.4.2: LDL-cholesterol inhibits Vγ9Vδ2 T cell activation and cytokine production 36

FIGURE 2.4.3: Pre-expanded γδ (~80% Vδ2+) T cells were cultured in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours 38

FIGURE 2.4.4: LDL-cholesterol limits the anti-tumour therapeutic effect of human γδ T cells in vivo 41

TABLE INDEX

Chapter 1

GENERAL INTRODUCTION

TABLE 1: γδTCR repretoire 4

ACKNOWLEDGEMENTS /AGRADECIMENTOS

Ao meu filho, Ângelo, pelo amor incondicional, pela compreensão e por ser a força que alavancou a concretização deste percurso.

Aos meus queridos pais, Hélder e Ângela (Ruth), pela confiança, pelo incentivo, pelo apoio incondicional e sacrifícios que fizeram e que ainda fazem para que tenha uma boa educação.

Aos meus familiares, Nair, Florimundo Carla, Edna e Carlos, pelo apoio incondicional, pela ajuda, pelo amor. Agradeço a todos indistintamente, pois, a vossa contribuição incansável foi fundamental para concretizar o percurso com sucesso.

Ao meu companheiro, Paulo Miranda, pelo carinho, compreensão e apoio incondicional para ultrapassar vários obstáculos ao longo deste percurso.

Ao Governo de Cabo Verde, a Fundação de Ciência e Tecnologia, a Fundação Calouste Gulbenkian, pelo financiamento ao programa PGCD, permitindo assim, que África dê os primeiros passos para criar quadro profissional de qualidade e também incutir a investigação científica em Africa.

A Doutora Joana Sá e a Carla Semedo, por aceitarem o desafio de coordenarem o programa PGCD, programa este que me deu a oportunidade de fazer parte do primeiro programa doutoral africana. Contudo, agradecer-vós em palavras é muito pouco, pelo todo o ensinamento, aprendizado, paciência e confiança que depositaram nos alunos do PGCD.

Ao Instituto de Medicina Molecular (IMM), a direção, a administração, aos técnicos dos serviços, pelo apoio, suporte e disponibilização dos serviços de qualidade que permitiram o desenvolvimento do projeto doutoral.

Aos meus orientadores, Professores Investigadores, Dr. Sérgio Dias e Dr. Bruno Silva-Santos, pela oportunidade, paciência, ensinamento, compreensão, pela confiança para fazer parte da vossa equipa, pela orientação na escolha e execução do projeto de investigação, e por proporcionarem-me um conhecimento, mas amplo sobre as diretrizes de uma investigação científica.

Aos meus colegas do PGCD, em especial, a Cíntia Ramos, o Viriato e o Daniel Zacarias, pelo eterno orgulho da nossa caminhada, pelo apoio, cumplicidade, paciência e amizade sincera que levarei para a vida toda.

Aos colegas do laboratório Sérgio Dias e Bruno Silva-Santos, pela receção amigável, pela ajuda na realização de trabalhos, pelos inputs, em especial, o Daniel Correia, que considero mais do que um colega, mas sim, um grande companheiro que me apoiou, e foi fundamental para que o projeto fosse levado a cabo.

As minhas colegas, Sandrina Nóbrega, Sofia Mensurado, Ana Magalhães, pelo vosso apoio, cumplicidade, e por todo carinho ao longo deste percurso.

A Natacha, pela toda paciência, apoio e profissionalismo que sempre demonstrou.

A todos, que diretamente ou indiretamente apoiaram, incentivaram, contribuíram, e acreditaram na concretização deste percurso, deixo aqui o meu sincero agradecimento.

"Apreendi que o doutoramento é mais que um percurso académico. Todavia, atrevo a descrevê-la, como um dos maiores desafios académico na vida de um investigador.
Durante o meu doutoramento, passei por várias situações delicadas, como, divórcio, um filho especial, dificuldades financeiras, doenças oncológicas familiares, perdas de entes queridos, entre outras situações, contudo, estas situações colmataram para que desenvolvesse resiliência emocional, que me ajudou a lidar com os obstáculos e manter o foco no trabalho de investigação. Em suma, queria demonstrar que ao longo dos quatro anos houve diversas situações que podia-me levar a desistência do percurso, porém, eu escolhi lutar por mim, pelo meu filho, pelos meus familiares e por todos os africanos que sonham em fazer ciência de qualidade na África. Por isso, espero que a minha história, sirva como fonte de inspiração e força para todas as mulheres, mães e investigadoras africanas."

ABREVIATIONS

Apo	Apolipoproteins
ATP	Adenosine triphosphate
CD	Cluster of Differentiation
CD3	CD3 molecule, (CD3-TCR complex)
CD4	Monomeric IgG superfamily protein expressed on CD4+ T cells
CD8	Homo or Heterodimeric protein co-receptor expressed on CD8+ T cells
CD69	Cluster of Differentiation 69
CTL(s)	Cytotoxic T lymphocyte(s)
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DNAM-1	DNAX accessory molecule-1
DNA	Deoxyribonucleic Acid
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HDL	High-density lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-10	Interleukin 10
IL- 17	Interleukin- 17
IL-2R	Interleukin-2 Receptor
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein Receptor
IPP	Isopentenyl pyrophosphate
IP	Interferon Gamma-Induced Protein 10
FCS	Fetal Calf Serum
FOXP3	Forkhead box P3
FITC	Fluorescein
LDL-R	LDL receptor
LFA-1	Lymphocyte function-associated antigen 1
LXRα	Liver X receptor alpha

LXRβ	Liver X receptor beta
MFI	Median Fluoresence Intensity
MHC	Major Histocompatibility Complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MULT1	Murine UL16 binding protein-like transcript
mRNA	Messenger RNA
NFAT	Nuclear Factor of Activated T Cells
NF-Kb	Nuclear Factor Kappa B
NKG2D	Natural-killer group 2, member D
NK	Natural Killer
NKR(s)	Natural Killer cell–associated receptor(s)
NKT	Natural Killer T
OKT3	Anti-CD3 antibody, clone OKT3
PBL(s)	Peripheral Blood Lymphocyte(s)
PBMC(s)	Peripheral Blood Mononuclear Cell(s)
PD1	Programmed cell death protein 1,
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
Pen/Strep	Penicillin Streptomycin
PerCP	Peridinin chlorophyll
PLCγ1	Phospholipase Cγ1
PerCP/Cy5.5	Peridinin chlorophyll-Cy5.5
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinases
RAG	Recombination activating gene
Rag	Recombination activating gene
RNA	Ribonucleic Acid

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute cell culture medium
RT-qPCR	Real-Time –quantitative Polymerase Chain Reaction
TCR(s)	T Cell Receptor(s)
Tet1	Ten-eleven translocation methylcytosine dioxygenase 1
TIL(s)	Tumor infiltrating lymphocyte(s)
TGF-β	Transforming Growth Factor Beta
Th1	T helper cell type 1: defined by the ability to produce Interferon-gamma
TLR(s)	Toll-like receptor(s)
TLR(s)	Toll-like receptor(s)
TNF	Tumor Necrosis Factor
TNFR	Tumor necrosis factor
ULBP(s)	UL16 binding protein(s)
UV	Ultraviolet
ULBP1	UL16 binding protein 1
ULBP4	UL16 binding protein 4
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa

Sumário

O microambiente tumoral é composto por diferentes tipos de células, células tumorais, e outras células tais como endotélio, fibroblastos, células estromais e subgrupos de leucócitos, incluindo linfócitos T γδ (gama delta), que exercem um papel importante no controlo da progressão tumoral [1–5]. Atualmente, uma das estratégias da imunoterapia para o cancro utilizando células T γδ é baseada na reatividade única do subtipo Vγ9Vδ2, dominante no sangue periférico, a antígénios de origem não peptídica (fosfoantígénios). Neste trabalho estudámos o papel do colesterol sistémico, e em particular o colesterol-LDL, na mobilização seletiva e na função efetora de células T Vγ9Vδ2 no cancro de mama. Os nossos resultados mostram claramente que o colesterol-LDL foi internalizado pelas células T Vγ9Vδ2, seguido pela internalização e regulação negativa da expressão do receptor de LDL (LDL-R), o principal receptor de lipoproteína transportadora de colesterol. Posteriormente, utilizamos plasma humano para confirmar o efeito do colesterol-LDL nas funções das células T Vγ9Vδ2, nomeadamente na diminuição da sua ativação mediada por fosfoantigénios. Avaliámos vários parâmetros de modo a compreender o papel do colesterol-LDL na viabilidade metabólica das células T Vγ9Vδ2. Os nossos resultados mostram que na presença de colesterol-LDL, as funções metabólicas das células T γδ foram significativamente inibidas no que respeita a: massa mitocondrial, DNA mitocondrial (mtDNA), ATP (Adenosina trifosfato) e espécies reativas de oxigénio (ROS).

Após elucidação da redução da viabilidade metabólica e ativação das células T Vγ9Vδ2 expostas a colesterol-LDL, concentrámo-nos nos mecanismos envolvidos no reconhecimento e interação com as células tumorais de cancro da mama. Entre os potenciais receptores citotóxicos, demonstrámos que a expressão de NKG2D e DNAM-1, envolvidos no reconhecimento de ligandos tumorais, bem como da potente citocina anti-tumoral, interferão-gama, foram diminuídas mediante a exposição a colesterol-LDL, reduzindo, consequentemente, a capacidade anti-tumoral das células T Vγ9Vδ2.

Foram realizados ainda estudos *in vivo* para esclarecer o papel do colesterol-LDL nas funções das células T Vγ9Vδ2 num contexto tumoral. Numa primeira

abordagem, avaliamos a capacidade antitumoral de células T Vγ9Vδ2 previamente incubadas na presença ou ausência de colesterol-LDL em ratinhos imunodeficientes com tumores de mama. Por outro lado, como a obesidade é caracterizada pela superprodução de citocinas inflamatórias, como TNF-alfa e interleucina-1, que podem levar à ativação de macrófagos, foi utilizada uma segunda abordagem com uma dieta rica em colesterol em ratinhos imunocompetentes para avaliar o impacto do microambiente rico em colesterol nas funções dos linfócitos T γδ e na progressão tumoral. Em ambas as experiências o impacto negativo do colesterol-LDL sobre as funções antitumorais dos linfócitos T γδ foi confirmado, levando a um maior crescimento do tumor.

O nosso estudo descreve, pela primeira vez, o papel do colesterol-LDL como importante inibidor das funções anti-tumorais das células T γδ. Assim, os nossos resultados sugerem que o "mau" colesterol, quando em altos níveis, terão um efeito inibitório sobre as funções das células T Vγ9Vδ2 que são normalmente mobilizadas para combater células tumorais. Esta nova descoberta pode ajudar no desenvolvimento do tratamento do cancro com imunoterapia, evitando que barreiras como níveis elevados de colesterol sejam desencadeadas, e assim preservando a ativação linfocitária necessária para eliminar as células tumorais.

Palavras-chave: células T Vγ9Vδ2; fosfoantigénios; colesterol-LDL, recetores de citotoxicidade; cancro de mama; imunoterapia.

SUMMARY

The tumor microenvironment is composed of different cell types, such as cancer cells, endothelial, fibroblast, stromal cells, and also leukocytes, including γδ T lymphocytes, which participate in anti-tumor immunity. Currently the main strategy for immunotherapy using gamma delta (γδ) T cells is based on the unique reactivity of its dominant subtype in the peripheral blood, Vgamma9Vdelta2 T cells (Vγ9Vδ2 T cells), to antigens of non-peptidic origin (phosphoantigens). Here we investigated the role of systemic cholesterol, namely LDL-cholesterol, in the selective activation and effector functions of T Vγ9Vδ2 cells, particularly in breast cancer. Our results clearly show that LDL-cholesterol was internalized by Vγ9Vδ2 T cells, which was followed by the down-regulation of the LDL receptor (LDL-R), the major cholesterol-carrying lipoprotein receptor. Thereafter, we used human plasma to confirm the effect of the LDL-cholesterol on Vγ9Vδ2 T cell functions. The role of metabolic changes within the immune microenvironment is a timely topic now that there are a variety of cellular therapies utilized to treat cancer patients. In order to understand the role of LDL-cholesterol in the metabolic viability of Vγ9Vδ2 T cells, several parameters were evaluated. In the presence of LDL-cholesterol, the metabolic functions of γδ T cells were significantly inhibited as shown by decreases in mitochondrial mass, mitochondrial DNA, Adenosine triphosphate (ATP) and reactive oxygen species (ROS).

Upon elucidation of the decreased metabolic viability involved in the negative regulation of Vγ9Vδ2 T cell activation by LDL-cholesterol, we next dissected the mechanisms involved in the recognition and interaction with tumor cells. Among the potential cytotoxic receptors, we demonstrated that the expression of Natural Killer group 2D (NKG2D) and DNAX Accessory Molecule-1 (DNAM-1), involved in the recognition of tumor cell ligands, and the potent anti-tumor cytokine interferon gamma (IFN-g), were all impaired in the presence of LDL-cholesterol, thereby affecting the cytotoxic capacity of the Vγ9Vδ2 T cells against breast cancer cells *in vitro*. Consequently, we conducted *in vivo* studies to support these findings in a tumor context. As a first approach, we evaluated the anti-tumor capacity of Vγ9Vδ2 T cells previously incubated in the presence or absence of LDL-cholesterol

in immunodeficient mice bearing human breast tumors. On the other hand, since obesity is characterized by overproduction of inflammatory cytokines, such as TNF alpha and interleukin-1, which may lead to macrophage activation, we used a second approach to evaluate the impact of a cholesterol-rich microenvironment on the antitumor functions of murine γδ lymphocytes in a syngeneic breast cancer model. In both experiments, the negative impact of LDL-cholesterol on the antitumor functions of γδ T lymphocytes was confirmed, leading to increased tumor growth.

The influence of cholesterol on tumor growth had been demonstrated in previous studies, where mice on a fat diet developed larger tumors compared to mice exposed to "normal" diets. One of the possible explanations offered by our study is that "bad" cholesterol, when at high levels, has an inhibitory effect on γδ T cells that are normally mobilized to destroy tumor cells. Moreover, our work is the first to describe how human Vγ9Vδ2 T cells may be suppressed by high LDL-cholesterol. This new finding can help in the development of immunotherapy against breast cancer, namely towards preserving the lymphocyte activation and functions required to eliminate tumor cells.

Keywords: Vγ9Vδ2 T cells; phosphoantigens; LDL-cholesterol, cytotoxicity receptors; breast cancer; immunotherapy.

1-GENERAL INTRODUCTION

1.1 Hallmarks of cancer

Breast cancer (BC) is the most common cancer among women worldwide[6]. Early detection and improved treatment have led to an increase in overall survival rate[7], however, many women are still diagnosed after tumor progression and in these cases, the overall survival rate decreases drastically.

According to Hanahan and Weinberg, genetic alterations or mutations are one of the six original "hallmarks" of cancer: 1-self-sufficiency in growth signals; 2-insensitive to anti-growth signals (tumor cells ignore anti-growth signals), 3-tissue invasion and metastasis; 4-limitless replicative potential; 5-sustained angiogenesis[8] and and 6-immune modulation [9].

Tumor relapse represents the main cause of death from BC, making the identification of biomarkers that could predict tumor behavior a major unmet clinical issue. Recently, four new hallmarks of cancer were revealed by the same authors, including genome instability[10], deregulation of cellular energetics[11], tumor promoting inflammation[12] and evading immune destruction[13]. Growing evidence shows that metabolic contents support tumor growth and metastasis by metabolic reprogramming and promotion of angiogenesis[14,15]. Altered lipid metabolism is being increasingly recognized as playing an important role in tumor onset and progression[14,16,17]. Most cancers are able to relapse by gaining limitless self-renewal, this hallmark of cancer being related to lipid metabolic reprogramming. This metabolic change is modulated by oncogenic signaling pathways and is important for the initiation and progression of tumors, since cellular growth is dependent on the sustained availability of lipids[14].

1.1.2 The tumor immunosurveillance hypothesis/theory

Understanding the mechanisms involved in tumor growth is essential for the development of new strategies, especially for more advanced diseases for which the current options have limited impact. According to Paul Ehrlich (in 1909), the immune system plays a crucial role against neoplastic cells, and he proposed that this was the reason why cancer cells are not stronger. Later, Lewis Thomas introduced the hypothesis (or theory) of immunological surveillance or immunosurveillance, which was subsequently developed by Burnet that suggested that the main evolutionary advantage of cellular immunity would be the control of neoplastic growth in multicellular organisms. Curiously, anti-tumor immunology is based on the fact that there are tumors antigens that can be recognized by the immune cells and can generate a response against neoplastic cells. However, experiments conducted in the 1940s in rats immunized with irradiated tumor cells demonstrated resistance of the antitumor response, suggesting that resistance could be mediated primarily by lymphocytes and the tumor rejection antigens are specific to individual tumors [18]. From the 1990s, new experimental models based on the use of transgenic animals and monoclonal antibodies allowed a more accurate dissection of the interaction between the immune system and cancer. After this, tumor immunology research intensified, with the demonstration that transplanted tumors grew more robustly in mice treated with neutralizing monoclonal antibodies specific for interferon-gamma (IFN-γ)[19], a key molecular component of the immune system [19,20]. This was reinforced by the observation of Kaplan et al., that tumor formation induced by the inoculation of the carcinogen methylcholanthrene (MCA) in immunodeficient mice that lacked either IFN-γ responsiveness or an intact T cell compartment [20,21]. Since then, several human and mouse tumor antigens that are recognized by the host immune system have been identified. For example, the role of the adaptive immune system in suppressing tumor growth was revealed by using mice without the Rag2 gene (recombinase-activating gene 2), which are unable to initiate gene rearrangement for the production of specific T cell receptors and immunoglobulins; and therefore, are B, T and NKT cell-deficient[22]. More recently, the role of Natural killer cells (NK cells) in immunosurveillance was highlighted by the protective role of the NKG2D

activation receptor, the absence of which increases the incidence of induced tumors, and its presence mimics the effects of perforin[23]. Moreover, an important role in immunovigilance was observed for γδ and αβ T cell-deficient mice, where tumors were induced by dimethylbenzanthracene and tetradecanoylforbolacetate [24].

1.1.3 Tumor infiltrating lymphocytes

Tumor relapse represents the main cause of death from BC making the identification of biomarkers that could predict tumor behavior a major issue in this pathology. Tumor infiltration by natural killer cell (NK cell), T cells and macrophages are associated with different prognosis of several tumors and impact tumor development[25]. While tumor-infiltrating T cells are generally associated with good prognosis, myeloid cells emerge as opposite predictors of survival for diverse solid tumors[26,27]. Growing evidence shows that myeloid cells support tumor growth and metastasis by suppressing anti-tumor T cells and promoting angiogenesis[28]. However, in some circumstances lymphoid subsets are subverted from their original tumor effector functions and *are actively"moving".* Recent studies have also shown that quantification of tumor-infiltrating FOXP3+ regulatory T cells (Tregs) is a novel marker for identifying high-risk BC patients and is valuable for assessing disease prognosis and progression[25,29]. Prognosis of tumor infiltrating lymphocytes in melanoma, epithelial ovarian cancer and colorectal patients was observed but with low CD8+ T cell infiltration [30,31]. It seems that the infiltration or presence of infiltrated T cells is not an indication of patient survival, but can be an indicator of enhanced survival in patients undergoing immunotherapy.

1.2 Gamma delta (γδ) T cells

γδ T cells, represent a small subset of T cells that possess a distinct T-cell receptor (TCR) on their surface composed of γ and δ chains. In humans, two major subsets of γδ T cells, Vδ1 and Vδ2, are defined based of δ chain usage. They are a minor fraction of the T lymphocytes in the peripheral blood (1-5%), are negative

for CD4 or CD8 co-receptors, and are found rarely in the lymph nodes and spleen, whereas, by contrast, they are abundant in the intestines, skin, tongue, esophagus, trachea, lungs and genital epithelia[32,33], where they play protective roles against intracellular pathogens and cellular transformation[2,3].

γδ T cells do not recognize MHC-peptide complexes as conventional T cells; they seemingly recognize molecules that are expressed on infected or transformed cells independently of MHC presentation. However, the conditions that lead γδ T cells to antigen recognition, activation and function are not fully understood. Nonetheless, γδ T cells are currently viewed as the 'first line of defense' or 'bridge "between innate and adaptive responses.

T cell responses depend on direct contact with the antigen presenting cell or the target cell, but the role of TCR ligands in γδ T cell activation remains controversial [2,34]. In humans, the major (50-95%) γδ T cell subset present in the peripheral blood expresses a TCR composed of Vγ9 and Vδ2 chains that is uniquely reactive to non-peptidic phosphoantigens .

Table 1: γδ TCR repertoires. Adapted from Bonneville *et al.*, 2010.

Species	**Peripheral Location**	**Predominant V gene segment usage**	**V (D)J diversity**
Mouse	Adult thymus Spleen Lymph nodes Epidermis Liver Gut epithelia Uterovaginal epithelia Lung epithelia	Diverse Vγ1 and Vγ4 Diverse Vγ5Vδ1 Vγ1Vδ6.3, Vγ4 and Vγ6 Vγ7Vδ4, Vγ7Vδ5 and Vγ7Vδ6 Vγ4 and Vγ6	High High High Invariant Intermediate Intermediate Invariant Intermediate
Human	Thymus Peripheral blood Spleen Liver Gut epithelia Dermis	Vδ1 Vγ9Vδ2 Vδ1 Vδ1 and Vδ3 Vδ1 and Vδ3 Vδ1	High Intermediate High High High High

1.2.1. Vγ9Vδ2 T cells in immunity

Vγ9Vδ2 T (Vgamma9Vdelta2) cells have been considered primate specific, as they do not exist in mice, but they can also be found in other species such as the alpaca. Four maturation stages of subsets of Vγ9Vδ2 T cells have been identified based on their phenotypes: the CD45RA+CD27+ and CD45RA−CD27+ subsets preferentially express the CD62L and CCR7 lymph node homing receptors; they are defined as naive (N) and central memory (CM) cells, respectively. In addition, the CD45RA−CD27− and CD45RA+CD27− subsets are negative for CD62L and CCR7 and instead express receptors for inflammatory chemokines, such as CCR5 and CXCR3[35–39].

They have several innate cell-like attributes that allow for their early and rapid activation following recognition of conserved stress-induced ligands [2], especially phosphoantigens[40,41]. Activated Vγ9Vδ2 T cells express high levels of cytotoxic mediators (granzymes, perforin, TRAIL, RANTES, IP-10 (Interferon Gamma-Induced Protein10), lymphotactin) and produce large amounts of IFN-gamma and TNF-α (Tumor necrosis factor alpha). In addition, other costimulatory receptors affect (CD2, CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4), ICOS (Inducible T-cell COStimulator) and PD-1 (Programmed cell death protein 1)) T cell activation, division, survival and cytokine production[42]. However, the contribution of γδ TCRs to such responses is unclear. Recently Benjamin and Carries discussed the identity of the several ligands recognized by the γδ T cell antigen receptor: TCR of a human V(γ)4V(δ)5 clone directly bound endothelial protein C receptor (EPCR)[43]; antigenic ligands of γδ TCRs, annexin A2 was identified as the direct ligand of Vγ8Vδ3 TCR[44]; Vgamma1.3Vdelta2-TCR, termed M88, recognizes various proteins from different species (including some bacterial and human aminoacyl-tRNA synthetases (AA-RS)[45]; Ephrin ligand binding domain Vγ9Vδ1 TCR[46]; the increased expression of T22 and/or T10 might trigger immunoregulatory gamma delta T cells during immune responses[46]; CD1c tetramers carrying Mycobacterium tuberculosis phosphomycoketide bind γδ TCRs[47]. Vγ9Vδ2 T-cell activation can be abrogated by exposing susceptible cells

(tumor and mycobacteria-infected cells, or aminobisphosphonate-treated cells with up-regulated PAg levels) ton antibody 103.2 against CD277 to antibody 103.2 against CD277[48].

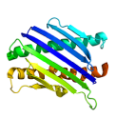
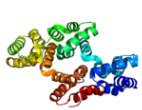
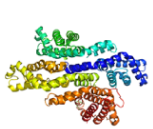
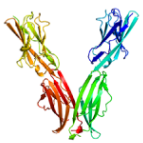
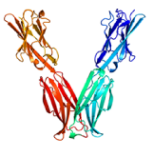
Name	EPCR	Annexin A2	tRNA synthetases	EphA2	T10 & T22	R-phycoerythrin	CD1c	CD1d	Skint-1	BTN3A	BTNL1–BTNL6 & BTNL3–BTNL8
Structure											
Homology	α1-α2-like	Annexin	Class II synthetases	Ephrin ligand binding domain	MHC class I–like	Phycobiliprotein family	MHC class I–like	MHC class I–like	B7-like	B7-like	B7-like
T cell population	$V_\gamma4V_\delta5^+$ (human)	$V_\delta3^+$ (human)	$V_\gamma3V_\delta2^+$ (human)	$V_\gamma9V_\delta1^+$ (human)	Diverse (mouse)	$V_\delta1^+$ (human)	$V_\delta1^+$ (human)	$V_\delta1^+$ (human)	$V_\gamma5V_\delta1^+$ (mouse)	$V_\gamma9V_\delta2^+$ (human)	$V_\gamma7^+$ (mouse), $V_\gamma4^+$ (human)
Frequency	25% of PB T cells (single donor)	ND	Majority of muscle-infiltrating T cells (single patient)	ND	<0.1% of splenocytes, <2% of IELs	<0.025% of total PB $CD3^+$ T cells	0.16% of total PB T cells	<0.05% of total PB T cells	>90% of epidermal T cells	~80% of PB γδ T cells	Proportion of intestinal γδ T cells
Differentiation status	$T_{effector}$ ($CD28^-$, $CD45RO^-$)	ND	$T_{effector}$ (cytotoxic)	ND	ND	ND	ND	ND	DETC phenotype	Typically $CD45RO^+$ $CD27^+$	ND
Affinity (if confirmed)	90 μM	3 μM	ND	Unconfirmed candidate	0.1 μM (mouse)	2.7 μM (mouse), ND (human)	23–150 μM depending on lipid	16–33 μM (loaded) 240 μM (empty)	Unconfirmed candidate	Unconfirmed candidate	Unconfirmed candidates
CDR3 involvement	CDR3γ and CDR3δ involved	ND	CDR3γ and CDR3δ involved	CDR3δ involved	CDR3δ involved (mouse)	CDR3 involved (mouse)	CDR3 involved	CDR3 involved	Unclear	CDR1, CDR2, CDR3 loops linked to p-Ag responses	Germline-encoded TCR regions linked to BTNL-mediated response

Figure 1: Ligands recognized by the γδ T cell antigen receptor. Adapted from Willcox, Carrie R. *et al.,* 2019.

1.2.2. Recognition of non-peptide antigens by human Vγ9Vδ2 T cells

In humans, Vγ9Vδ2 T cells respond to self and non-self-molecules and the microbial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) also known as phosphoantigens (pAgs), which is produced by pathogens[5]. HMB-PP is a, physiological activator and intermediate of the non-mevalonate pathway of isoprenoid biosynthesis of many eubacteria and plants. This most potent natural phosphoantigen was first characterized from *E. coli*[49]. It was demonstrated that HMB-PP is potent activator of Vγ2Vδ2 T cells in response to several infections, like malaria and Tuberculosis[50]. HMPP is the immediate precursor of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and is a phosphorylated intermediate of the isoprenoid biosynthesis pathway, produced by eubacteria and protozoa but not by eukaryotes[49]. T cells recognize accumulation of IPP in transform cells or cells that accumulate IPP[51]. It was found that aminobisphosphonates such as zoledronate or pamidronate used for myeloma therapy or osteoporosis inhibit the IPP- consuming enzymes of isoprenoid biosynthesis, resulting in an increase in high numbers of blood-borne γδ T cells[52]. Vγ9Vδ2 T cells can recognize HMBPP phosphoantigen on the APC surface, clonal expansion of Vγ2Vδ2 T cells in response to signals from TCR[41,53] and cytokines [54], immune responses of HMBPP-activated Vγ2Vδ2 T cells[55], T effector functions to cytokine production, and trafficking of Vγ2Vδ2 T cells to the lungs or mucosal surfaces [56]. As an example, it was demonstrated that pathogens such as *Toxoplasma gondii*, *Yersinia enterocolitica*, *Plasmodium falciparum and Mycobacterium are* not restricted to the recognition through MHC molecules by *Vγ9Vδ2 T cells*. The high potency of HMB-PP as a stimulator of Vγ9Vδ2 T cells correlates with the γδ T cell stimulatory activity of the bacteria (like Mycobacterium tuberculosis and E. coli) exploiting the MEP (2-C-methyl-D-erythritol 4-phosphate) but not the mevalonate pathway [55] (Figure 2).

In addition, it was shown that activation of Vγ9Vδ2 T cells requires expression of butyrophilin 3A1 (BTN3A1). BTN3A1, (BTN3; CD277) immunoglobulin superfamily proteins play a crucial role in the recognition process[57]. Nevertheless, the mechanism of activation of Vγ9/Vδ2 T cells by BTN3A molecules remain unknown [2,5,58,59].

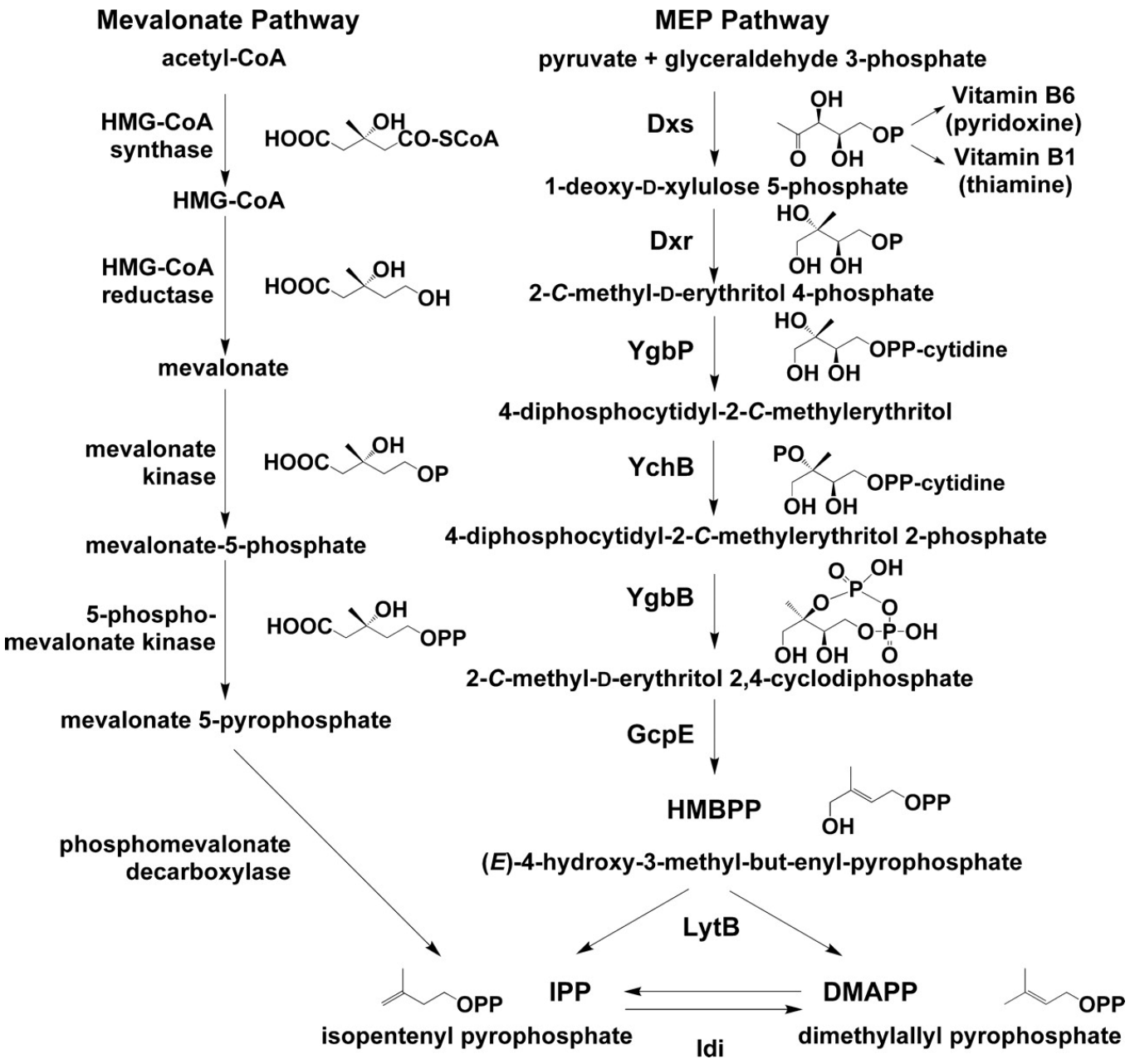


Figure 2: Mevalonate and MEP (2-C-methyl-D-erythritol 4-phosphate) pathways for isoprenoid biosynthesis. Adapted from Morita *et al.,* 2007

1.2.3. TCR-mediated activation

γδ T cell receptor (TCR) is composed of a γ and δ chain heterodimer, both products of V(D)J recombination, which distinguishes them from conventional αβ T cells[60]. Human TCR γδ T cells are independent of MHC Class II for cytotoxicity but require complex of invariant trans-membrane proteins called CD3 for function and cytotoxic activity[61]. TCR antigen recognition initiates a sequence of T-cell biochemical signals, MAPK/Erk and PI3 K/Akt pathways that results in the activation of transcription factors, NF-kB/NFAT, which control certain gene encoding proteins, Fos/Jun, that mediate the biological responses of these cells (figure 3)[62]. A complex of chains that are associated with the CD4/CD8 and CD3 molecules forms the TCR[63]. In summary, when TCR binds to the peptide-MHC complex, CD4 or CD8 bind to the MHC constant regions and the Lck enzyme (tyrosine kinase associated with each CD4/CD8 molecule) approaches CD3, leading to the ITAM phosphorylation (motifs activators based on tyrosine) present in these molecules. The phosphorylated ITAMs are binding sites for the zeta-chain-associated protein of 70 kDa (ZAP-70) molecule, which is activated and acts by phosphorylating several cytoplasmic signaling molecules that promote the activation of signal transduction pathways, such as the RAS-MAP kinases (Mitogen Activated Protein Kinase) and PCLγ1[64]. These pathways converge to activate transcription factors such as nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1) and nuclear factor kappaB (NFκB). NFAT is the transcription factor necessary for expression of cytokine genes (such as IL-2, IL-4, TNF), that is activated by calcineurin, which deforms it, allowing the NFAT to translate to the nucleus and to bind in the regulatory regions of the cytokine genes[65,66].

Co-stimulation signals (mainly via CD28) cooperate with TCR signals to increase the activation of transcription factors (eg, they activate NFκB to induce the production of IL-2 that promotes proliferation and survival of activated T lymphocyte)[67].

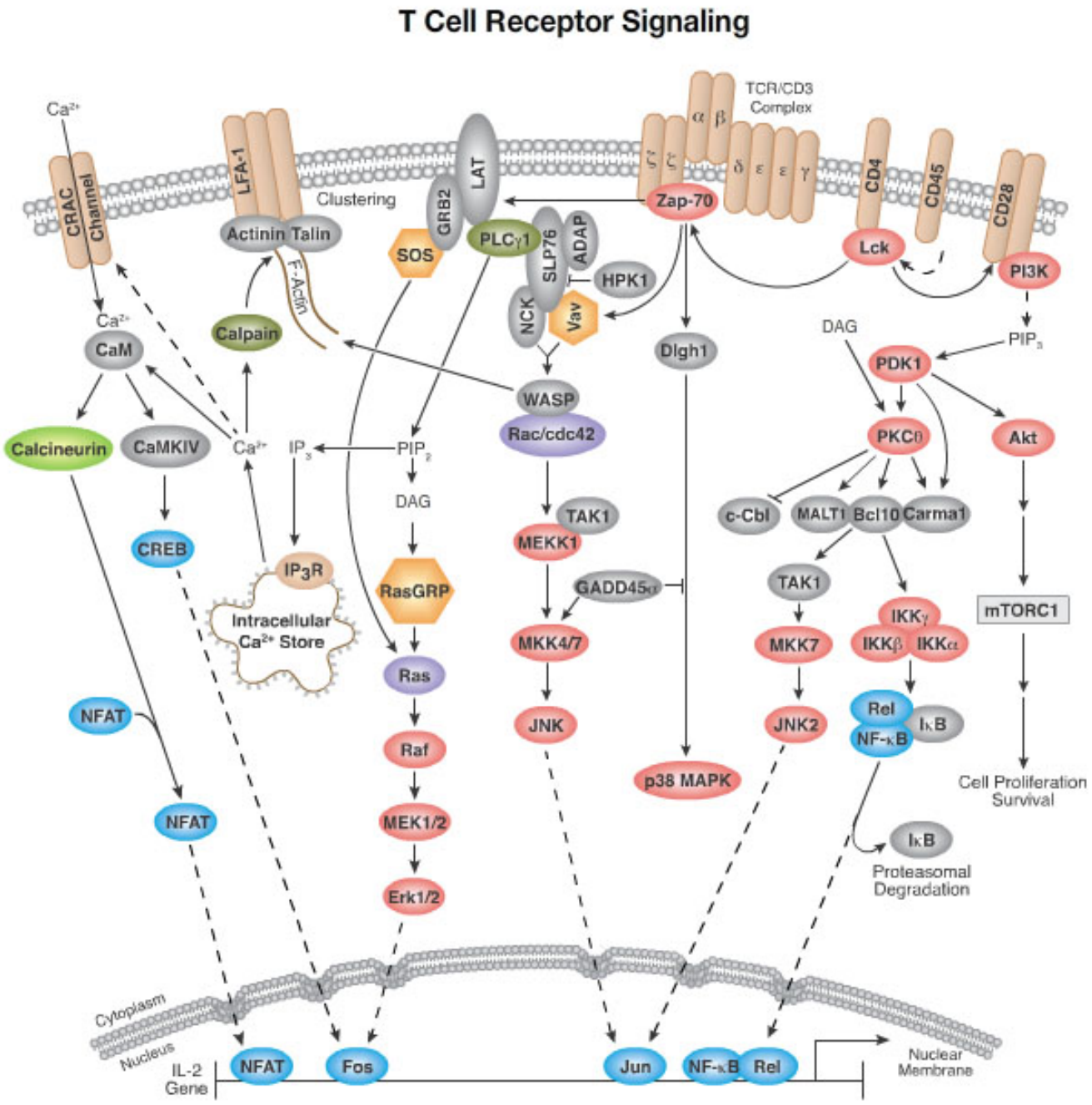


Figure 3: T cell receptor signaling cascade. Adapted from http://www.cellsignal.com/

1.2.4. Cytotoxic receptors

1.2.4.1. NGK2D

γδ T cells share many characteristics with NK cells, including the expression of NK receptors, in particular NKG2D. NKG2D is an activating receptor belonging to the CD94/NKG2 family of C-type lectin-like receptors, present in both mice and human[68]. In mice, multiple members of the structurally related retinoic acid early transcript 1 (RAE1), histocompatibility 60 families (H60) and Murine UL16 binding protein-like transcript (MULT1) have been described. NKG2D needs the DNAX (adaptor molecule) to initiate signaling transduction and cellular activation [69].

The role of NKG2D function in tumor immunity was demonstrated using experimental animal models [70]. NKG2D ligands (like MICA , MICB, and at least four related ULBP family proteins) are generally expressed at the surface of infected, stressed or transformed cells[71,72]. In addition, the overexpression of the NKG2D ligands ULBP1 and ULBP4 by human hematological and epithelial tumors, respectively, drives efficient cytotoxic responses by Vγ9Vδ2+ T cells. Nevertheless, human cancer cells are found to broadly express NKG2D ligands[69,70]. In vivo studies demonstrated that ectopic expression of NKG2D ligands on tumor cells is sufficient to cause tumor rejection[73,74]. Both studies, suggest that increasing NKG2D expression on T cells or NKG2D ligand on target cells should be a viable avenue for developing effective cancer immunotherapy.

1.2.4.2 DNAM-1 (CD226)

DNAX accessory molecule-1 (DNAM-1, also known as CD226), a transmembrane glycoprotein tightly associated with lymphocyte function-associated antigen 1 (LFA-1), is an activating receptor molecule expressed on the surface of NK cells, T lymphocytes, platelets, monocytes and a subset of B cells[75]. DNAM-1, was found to bind to nectin molecule CD112 (also known as nectin-2 or PRR2) and the nectin-like molecule CD155 (also known as poliovirus receptor PVR or Necl5), and it was demonstrated to be a pivotal regulator of NK functions against cancer[76,77]. In

recent years, results from a series of studies, have showed the involvement of DNAM-1 in intercellular adhesion, lymphocyte signaling, cytokine secretion and cytotoxicity of T lymphocytes induced by cytotoxic NK cells and T lymphocytes[72,77]. Both NKG2D and DNAM-1 ligands expressed in target cells initiate a similar intrinsic response to cellular stress[78] and the induction of DNAM-1 receptor expression has already been used as a target for anti-cancer immunotherapies [79].

1.2.5. V9γVδ2 T cells in cancer immunotherapy

Increasing evidence suggests that immunotherapy is a promising approach for treating patients with invasive and metastatic breast cancer[4] . In fact, there is still a real need for new therapies for human epidermal growth factor receptor 2 (HER2+) BC patients, especially those with resistance to trastuzumab, and further molecular characterization of these tumors is warranted in order to develop successful alternative therapeutic strategies. The ability of γδ T lymphocytes to produce abundant pro-inflammatory cytokines like IFN-γ, potent cytotoxic effector function and MHC-independent recognition of antigens makes it an important player in cancer immunotherapy[80]. Based on this knowledge, intravenous application of the N-BP zoledronate (or synthetic phosphoantigens like BrH-PP or HMB-PP) together with low-dose IL- 2 has been evaluated as a means of *in vivo* activation of γδ T cells in cancer patients[81,82]. Other clinical trials with aminobisphosphonates, drugs used to treat metastatic prostate cancer and breast cancer were observed in patients with partial remission[81,71]. In addition to this, complete remission was observed in a patient with metastatic renal cell carcinoma using adoptive transfer autologous in vitro-activated γδ T-cells plus low-dose interleukin-2 and zoledronic acid intravenous infusion[83].

Furthermore, other drugs such as bisphosphonates, especially nitrogen-containing bisphosphonates (NBPs) used extensively to treat osteoporosis and skeletal malignancies stimulate γδ T cell activation. NBPS drugs like zoledronate, alendronate and pamidronate, inhibit the key enzyme of the mevalonate pathway, and Farnesyl Pyrophosphate Synthase (FPPS), culminating in the upregulation of

the endogenous pool of IPP, which stimulates Vγ9Vδ2 T cells to release inflammatory cytokines[84,85].

Despite the fact that adoptive cell therapy has recently been proposed as an alternative therapeutic strategy to overcome or avoid resistance to drugs, clinical effectiveness and development of adverse side effects are still under investigation[86].

The immunity to cancer requires different types of cytokines. There are two types of cytokines that are already approved for anti-cancer treatment; type 1 interferon and interleukin 2[4]. γδ T cells can also be recovered from blood, expanded *ex vivo* and reinfused into patients at a later point in time. Future trials should harness bisphosphonate activated γδ T cells in combination with chemotherapy or monoclonal antibodies for treatment of solid tumors and hematological malignancies. Thus, γδ T cells are attractive for cell therapy strategies against cancer [4,87,88].

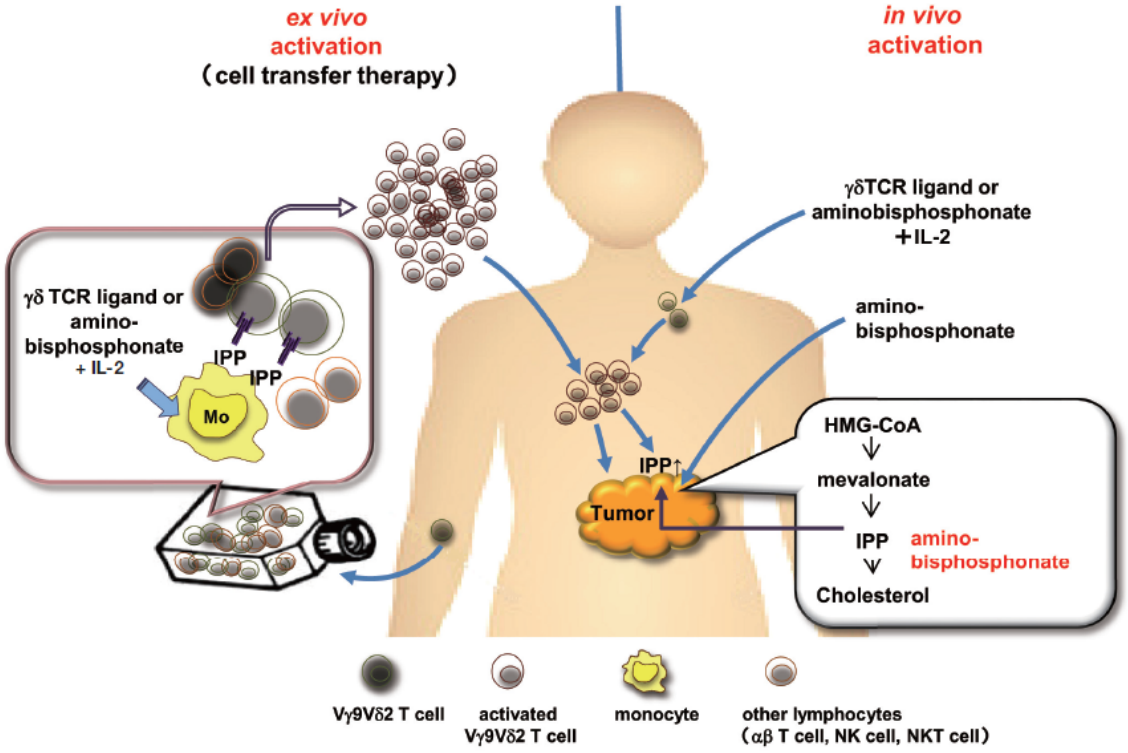


Figure 4: Strategies *in vivo* to administer compounds that activate γδ T cells or adoptive transfer of ex vivo expanded γδ T cells. Adapted from K, Kakimi *et al.*, 2014.

1.3- Metabolism and immune cell function.

Normally metabolism is seen as a way to store energy through catabolism or through anabolic pathways to generate macromolecules necessary for cell growth and viability. The elucidation of metabolic pathways have allowed a new understanding of disorders in which there are obvious dysfunctions in metabolism, such as atherosclerosis[89] and diabetes[90]. Changes in metabolic regulation are now also seen as of great importance in other diseases, including inflammatory conditions and cancer[91,92]. For instance, in the mid-1920s, the German physiologist Otto Warburg proposed that reprogramming of bioenergetics/metabolic deregulation is a special feature of cancer cells. Thus, understanding the interrelationship between metabolic machinery and cellular signaling has emerged as a new focus in the study of metabolic disorders, cancer and, more recently, in immune response.

Recent studies are demonstrating a close relationship between metabolic processes and the modulation of the immune cells response[93]. Tannahill *et al.*, demonstrated that succinate, a small dicarboxylic acid that is a metabolic intermediate in the Krebs cycle and substrate for the respiratory chain, modulates the function of hypoxia inducible factor 1 α (HIF-1α) that ultimately influences the synthesis of IL-1β by macrophages[94]. Moreover, in the presence of stress or pathogens, T cells need to adapt their cellular metabolism to maintain their effector and proliferative functions[95]. According to Pearce *et al.,* nutrients and other signaling molecules, such as cytokines, that are available in the environment can influence T cells function. An example is the fact that *de novo* fatty acid synthesis controls the fate between Treg cells and Th17 cells[96]. In addition, the depletion of glucose impairs T cell function and differentiation as well as interferon- γ (IFN- γ) production[97,98]. Other studies demonstrated that the exposure of dendritic cells to LPS leads to an increase in glucose consumption and lactate formation and a reduction in oxygen consumption[99].

Recent studies have shown great interest in the pathways, mediators and mechanisms that are involved in the interactions between metabolism and the immune system. Rapamycin (inhibits the mechanistic target of rapamycin, mTOR)

is considered a key-signaling pathway in controlling T cell metabolism. Studied have shown that modulation of the PI3K /mTOR axis is critical in the T lymphocyte differentiation process with slightly different roles in CD4+ and CD8+ T lymphocytes [100]. In both cell subtypes, inhibition of mTOR activity leads to a decrease in the effector phenotypes, but in CD4+ T cells an increase in both the development and differentiation of regulatory T cells was observed [101] whereas in CD8+ T cells it favors the memory phenotype [102].

1.3.1 Cellular cholesterol: physiological and homeostasis

Cholesterol is a lipophilic molecule essential for our body; it contributes to maintain structural cell membranes and regulates the fluidity of the membrane in various temperature ranges [103–105]. It is a precursor molecule for the synthesis of vitamin D and various steroid hormones, including cortisol and aldosterone in the adrenal glands and for the sex hormones progesterone, various estrogens, testosterone and its derivatives. Cholesterol is a fundamental part of the structure of bile salt [106] and participates in the absorption of fat-soluble metabolic vitamins, like vitamins A, D, E and K [107].

As cholesterol is not water soluble, the transport of this lipophilic molecule in circulation is made by hydrophobic proteins, lipoproteins (LP), whose structure is constituted by clusters of lipids molecules (cholesterol esters, triglycerides and phospholipids) and apolipoproteins that help in the transport of lipids through the body. There are several types of cholesterol, and they are generally classified according to their density: chylomicrons, VLDL (very low-density lipoprotein), IDL (intermediate-density lipoprotein), LDL (low-density lipoprotein) and HDL (high-density lipoprotein)[108]. Notably, LDL is the major lipoprotein responsible for cholesterol transport and stands out for its ability to bind to the cell membranes of epithelial cells that constitute the blood vessels. ApoB100 is the major apolipoprotein of LDL that *mediates* the binding LDL to its receptor. Differently, HDL plays an opposite role in reverse cholesterol transport back to the liver, the

only site of cholesterol excretion. In addition, ApoA1 is the major protein of HDL and is responsible for reduced risk of coronary diseases [109].

Mammalian cells acquire cholesterol molecules from the blood circulation in the form of plasma lipoproteins or intracellularly through the synthesis of cholesterol from the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, officially abbreviated HMGCR[103]. This enzyme catalyses the rate-limiting step in the mevalonate, squalene, and lanosterol pathway, the metabolic *pathway* that produces cholesterol and other isoprenoids and is the molecular target of statins[110].

The metabolism/regulation of LP is controlled by their Apolipoproteins (Apo), specific receptors such as the LDL receptor (LDL-R; that mediates the *catabolism of LDL and degradation of ApoB*)[111,112], ABCA1 (ATP-binding cassette transporter A1; transports excess cholesterol from cells to poorly lapidated pre β-HDL or apo-A1)[113], SR-A1 (Scavenger receptor A1; uptake of oxidized and modified LDL by macrophages) and the SR-B1 receptor (selective uptake of excess cholesterol from tissues into HDL as well as from HDL when it reaches the liver)[91,114]; enzymes such as Lipoprotein lipase (synthesized by adipose tissue and striated muscle), hepatic lipase (present in hepatocytes and transported to hepatic endothelial cells) and LCAT (synthesized by the liver and attached to HDL in blood); and, moreover, to transfer proteins [115].

LDL is bound and taken up by the specific LDL-R in the liver and many other tissues. The LDL-R protein is involved in receptor-mediated endocytosis of LDL, which is mediated by sterol regulatory element-binding proteins (SREBPs), situated in the ER membrane[116]. In mammalian cells, there are two different isoforms of SREBPS; SREBP 1 (variant SREBP 1a and 1c) and SREBP 2. SERP1 is responsible for regulating the genes required for *de novo* lipogenesis and SERBP 2 controls cholesterol metabolism, mediates lipids transport and lipoprotein uptake. However, endocytosis of LDL is a primary way for the body to decrease cholesterol levels so it follows that a decrease in LDL receptor function would also increase LDL concentrations in the blood. Studies have shown that a genetic anomalies in the LDL-R gene causes the autosomal dominant disorder familial hypercholesterolemia[117,118]. In addition, LDL-R and the cholesterol esterification enzymes (ACAT1/2) regulate the intracellular cholesterol levels, through

alteration of cholesterol internalization levels or by converting cholesterol into cholesterol esters[110]. It was also shown that inhibition of cholesterol transport with ACTA-1, the mitochondrial enzyme involved in the formation and degradation of fatty acid oxidation and amino acids, reduces lipid raft signaling pathways in CD8+ T cell[119] .

In contrast, excess of intracellular cholesterol is mediated by the ATB-binding cassette transporter A1 or G1 (ABCA1/ABCAG1), cholesteryl ester transfer protein and liver X receptors (LXR)[110]. The LXR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors, a pivotal regulator of lipid homeostasis in mammals. There are two isoforms of the receptor, LXRα (expressed in adipocytes) and LXRβ (expressed in the liver). Furthermore, immune cells express LXRβ than more LXRα. In the recent years, LXRs have been characterized as key for cholesterol transport, glucose metabolism and modulation of inflammatory responses. These findings led to identification of LXR in a variety of malignancies, such as atherosclerosis and diabetes[120,121].

Lipoproteins can be detected in the clinical setting to estimate the amount of cholesterol in the blood[122]. Hypercholesterolemia is considered a condition where patients have elevated cholesterol in the blood; causing a reduction in the uptake of LDL by the cells and, consequently, an increase in the concentration of LDL in the blood. In high concentrations in the blood, the LDL begins to deposit in the wall of the blood vessels, causing arteriosclerosis[115,123]. Conversely, LDL can be modified by oxidation (oxLDL), a complex process during which both the protein and the lipids undergo oxidative changes, and then are taken up by macrophages in the arterial intima resulting in the formation of foam cells, an important step in atherogenesis. This concept originated from a study where in vitro incubation of macrophages with oxidized but not with native LDL led to cholesterol ester accumulation[91,105,124] .

1.3.2 Cholesterol in immune cells

In human cells, cholesterol is an integral and very important part of the cell membrane. Different types of cells in our body need variable amounts of cholesterol, depending on their functions and purposes. If the cell is part of a protective barrier, for example, it will contain more cholesterol to make it robust and resistant to any invasion. If the cell, or organelle inside the cell, needs to be soft and fluid, it will contain less cholesterol in its structure.

As mentioned, the consumption of high-cholesterol diets can lead to increased levels of LDL, promote cholesterol accumulation in macrophages, and activate the inflammatory response in the artery wall; which drives the process of hypercholesterolemia and atherosclerosis[125]. It has previously been demonstrated that cholesterol accumulation in macrophages promote TLRs signaling activation, leading to cholesterol efflux, which causes more accumulation of cholesterol and stimulates inflammatory response [126]. In addition, obesity can directly trigger pro-inflammatory signaling pathways in adipose macrophages in response to lipids, which induces insulin resistance[127]. Moreover, cholesterol excess in haematopoietic stem cells (HSCs) increases the production *proliferation* of monocytes, which contributes to their accumulation in the atherosclerotic plaques[128]. It was demonstrated that in neutrophils, oxLDL and native LDL induce the upregulation of LOX-1 through activation of the ER stress response. However, the acquisition of immunosuppressive activity by neutrophils indicate that induction of ER stress response could convert neutrophils to Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) and the LOX-1 signal contributes to induce the suppressive activity of T cells functions[129]. Accordingly, human γδ T cells can recognize and interact with phospholipid antigens presented by CD1d, a non-polymorphic major histocompatibility complex class I-like antigen presenting molecule[125]. The recognition of lipid antigens presented by CD1d is usually associated with NKT cells[130]. NKT cell receptors (NKR) play a crucial role in the tumor recognition process by γδ T cells. Recently Yang et al, showed that in CD8+ T cell, cholesterol accumulation decreases SREBP activity and downregulates sterol synthesis and import, suggesting that LDL is internalized by T cells via LDL-R[119].

Although the mechanism by which LDL contributes to the T cell function in a cholesterol-rich environment and how its inhibition produces antitumor effect could be complex or unknown, it confirms the importance of cholesterol metabolism in immune cells. Recent studies in mice suggest that hypercholesterolemia induces the downregulation of Tet1 (Ten-eleven translocation methylcytosine dioxygenase 1) in hematopoietic stem cells leading to the reduction in cell number of NK and γδ T cells [131].

1.3.3 Cholesterol influence in TCR signaling/ T cells functions

TCR signaling promotes a number of signaling cascades that regulates T cell function, through specific cell surface receptor interactions and with soluble molecules, including cytokines, interferons, and chemokines. Therefore, cholesterol have been shown to play a role in T cell maturation, activation and immunological of synapse[132,133]. Contrary to memory cells, the naïve cells are cells that have not yet encountered pathogen. It has been shown that memory CD8 T cells respond more effectively to antigens than naïve CD8 T cells, and that cholesterol is the key factor in this process[134,135]. Peptide-selected thymocytes show similar phenotypes to mature CD8 + T cells and can respond to antigens[136].
Interaction between the CD3epsilon cytoplasmic domain of the TCR and phospholipids is required for control receptor activation[137]. Different data indicate that the fatty acids can modulate proliferation and function of T-cells[132,133,138]. It has also been demonstrated that, the activity of the αβ TCR is controlled by allosteric regulators, peptide-MHC and cholesterol-binding, which become spontaneously active[139,140]. In addition, mice administered with squalene, a cholesterol precursor were observed to have an increased number of CD4 T helper cells subsets in the periphery. Moreover, the authors suggest that the membrane cholesterol could be a new therapeutic target to modulate the immune function. Recently a study showed that cholesteryl esters play a role as cofactors in CD1c, allowing self-reactive T cells to bind to the protein[141]. In addition, cholesterol

sulfate (an inactive form of cholesterol.) can disrupt TCR multimers, inhibiting signal transduction well (Figure 5).

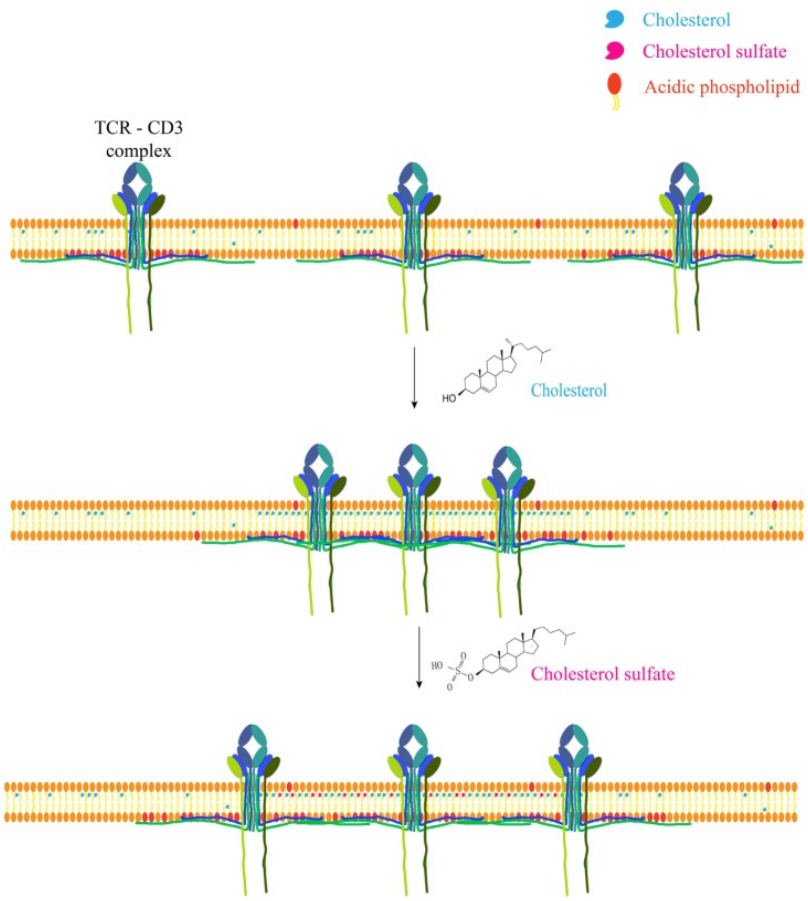


Figure 5: Cholesterol as signaling molecule in T Cells. Adapted from Andreas Bietz *et al.*, 2017.

1.4- Systemic cholesterol and tumor progression

During the last decade, the knowledge about the biology of BC has increased significantly. One of the main advances was the understanding of BC as a complex disease, composed of several clinical, morphological and molecular variables, including the expression of the estrogen receptor ("estrogen receptor, ER"), progesterone receptor, PR ") and the" human epidermal growth factor receptor 2, HER2". In recent years, the impact of the tumor microenvironment in tumor growth has gained outstanding interest from the oncobiology community [11,142]. The “systemic” capacity of a tumor to communicate with the surrounding

environment within the organism dictates its aggressive behavior. Such capacity includes the formation of neo-vessels (angiogenesis), evasion of immune responses and the formation of metastases [143]. Shifts in lipid metabolism were shown to drive tumor recurrence after therapeutic intervention [144]. Among the frequent alterations in lipid metabolism, *de novo* lipid biosynthesis has been suggested to play a major role in cancer pathogenesis [145]. There is emerging evidence that the uptake of exogenous lipids by tumor cells and by non-malignant cells in the tumor microenvironment may also contribute to malignancy [129,146,147]. Importantly, this particular metabolic feature has been pointed out as a possible explanation for the association of tumor progression, including BC, and high fat/high cholesterol diets [148,149]. Systemic modulation of metabolic pathways is, as mentioned above, a common feature of cancer. Aerobic glycolysis (the "Warburg effect") is one of the best known examples of tumor adaptation to (systemic) nutrient availability. However, tumors may perturb the "host" metabolism in other ways, including lipid synthesis. The outcome of such metabolic plasticity is the selection of tumor cells with increased nutrient independency and simultaneously with increased capacity to perturb the "host" metabolic needs. Moreover, systemic lipid levels and obesity have long been linked to increased cancer risk and to the development of more aggressive and more metastatic cancers

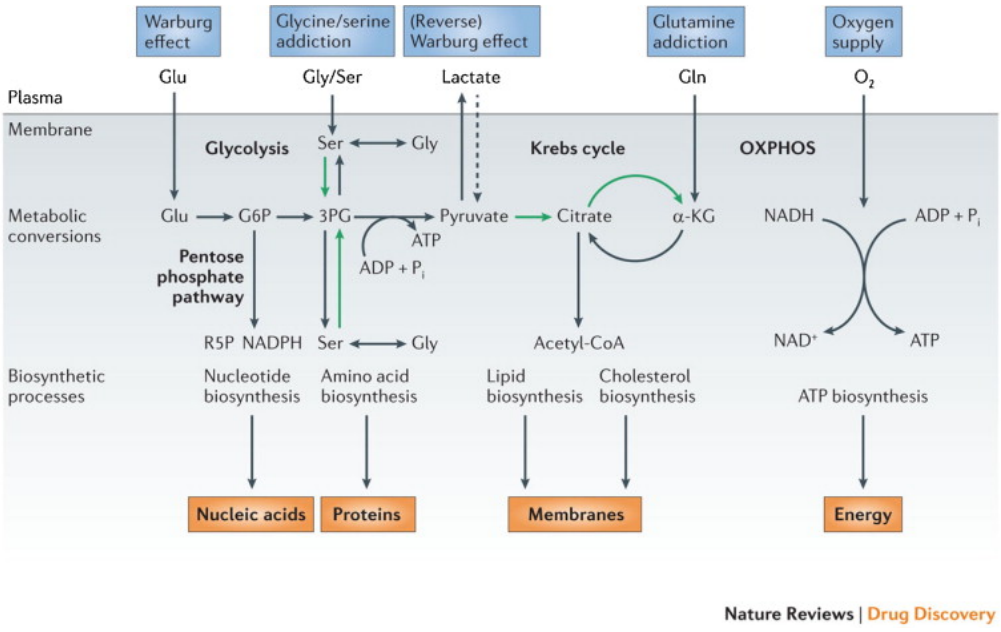


Figure 6: Lipid metabolism and Cancer progression. Adapted from Maxwell *et al.,* 2015.

1.5. Objectives of thesis

The main goal was to analyze anti-tumor proprieties of γδ T cells when exposed to LDL-cholesterol, evaluate their ability to internalize cholesterol and the effects on their level of activation and their ability to destroy breast cancer cells. For finally, we analyzed the effects of human plasma on the immune response of γδ T cells, in order to optimize their anti-tumor function, and thus provide new fundamental knowledge, development and implementation of innovative cancer immunotherapies based on the activation of gamma-delta T lymphocytes.

2-LDL-cholesterol uptake inhibits the activation and anti-tumor functions of human Vγ9Vδ2 T cells

2.1- Abstract

Vγ9Vδ2 T cells, the main subset of γδ T lymphocytes in the human peripheral blood, are endowed with potent anti-tumour functions such as cytotoxicity and IFN-γ production. These are triggered upon TCR-dependent activation by non-peptidic prenyl pyrophosphates ("phosphoantigens") that are selective agonists of Vγ9Vδ2 T cells, and have thus been evaluated in clinical studies. Since the latter have revealed a high degree of inter-individual variation in Vγ9Vδ2 T cell activities, we considered that metabolic resources, namely lipids like cholesterol, could affect phosphoantigen-mediated Vγ9Vδ2 T cell activation and functions. Consistent with this hypothesis, we show here that Vγ9Vδ2 T cells express the LDL-Receptor upon activation and thereby efficiently uptake LDL-cholesterol. This results in metabolic changes, such as decreased mitochondrial mass and reduced ATP production, that associate with downregulation of Vγ9Vδ2 T cell activation and functionality. In particular, the expression of IFN-γ, NKG2D and DNAM-1 are drastically reduced upon LDL-cholesterol treatment of phosphoantigen-expanded Vγ9Vδ2 T cells. As result, their capacity to target breast cancer cells is significantly compromised both in vitro and, especially, in an in vivo xenograft model. Thus, this study describes, to our knowledge for the first time, the role of LDL-cholesterol as a potent inhibitor of the anti-tumour functions of phosphoantigen-activated Vγ9Vδ2 T cells, which may have important implications for their therapeutic application

2.2 Introduction

Among the lymphocyte populations being considered for cancer immunotherapy are γδ T cells, which display key anti-tumour functions, like cytotoxicity and IFN-γ production, upon activation[1,2]. In humans and other primates, most (70-95%) γδ peripheral blood lymphocytes (PBL) express heterodimers of Vγ9 and Vδ2 chains. These Vγ9Vδ2 T cells have been shown to efficiently recognize and kill a variety of tumour cell types, such as melanoma, leukaemia, lymphoma, lung, ovary and

breast cancer; and, importantly, this does not require conventional antigen processing or MHC-mediated presentation[3,4]. Instead, Vγ9Vδ2 T cells are selectively activated by small non-peptidic prenyl-pyrophosphate metabolites of isoprenoid biosynthesis, termed phosphoantigens (PAgs), in a TCR-dependent but MHC-independent manner [5,6]. In fact, it was recently found that phosphoantigens critically interact with an intracellular domain of butyrophilin-3A1 (BTN3A1; CD277), a B7 superfamily member that seemingly provides the extracellular motifs recognized by Vγ9Vδ2 TCRs [7-9]. Interestingly, Vγ9Vδ2 TCRs are enriched for public Vγ9JP+ sequences[10] that may account for their 'innate-like' (i.e., fast) kinetics of response to PAgs. While the most potent PAgs derive from the non-mevalonate pathway of bacteria and parasites, tumour cells accumulate metabolite intermediates of the mevalonate pathway (e.g. isopentenyl pyrophosphate; IPP) that also activate primate Vγ9Vδ2 T cells [5,11,12].

Besides the TCR, also NK cell receptors, particularly NKG2D and DNAM-1, make important contributions to tumour cell recognition/ targeting by Vγ9Vδ2 T cells. Thus, we showed that NKG2D is critical for recognition of leukaemia and lymphoma cells by phosphoantigen-activated Vγ9Vδ2 T cells, with ULBP1 being a dominant NKG2D-ligand expressed on the target cells[13]. Other NKG2D-ligands, namely MICA [14] and ULBP4 [15], were implicated in NKG2D-mediated recognition of multiple myeloma, ovarian and colon carcinoma[1]. On the other hand, DNAM-1 ligands, Nectin-like-5 and Nectin-2, underlie hepatocellular carcinoma (HCC) cell targeting by Vγ9Vδ2 T cells [17]. We have therefore proposed that NK receptors are the key determinants of anti-tumour cytotoxicity of phosphoantigen-activated Vγ9Vδ2 T cells [18].

These anti-tumour properties of Vγ9Vδ2 T cells, together with their independence of MHC class I presentation (which tackles a common immune evasion mechanism) and presumably of mutated epitopes (thus making them ideal effectors against tumours with low mutation loads), have prompted their manipulation in the clinic[19]. Most clinical trials have used, as chemical means to selectively activate and expand Vγ9Vδ2 T cells, aminobisphosphonates like pamidronate and zoledronate, which are FDA-approved drugs for the treatment of osteoporosis and bone metastases. Notably, these drugs interfere with PAg-

processing enzymes and thereby increase the intracellular levels of IPP in tumour cells [20]. Alternatively, the synthetic PAg, bromohydrin pyrophosphate (BrHPP), has been employed either in vivo or ex vivo to activate/ expand autologous Vγ9Vδ2 T cells for re-infusion. However, the clinical performance of Vγ9Vδ2 T cells has been variable and somewhat disappointing thus far. Moreover, the prognostic value of Vγ9Vδ2 (or total γδ) T cell infiltration in tumours has also been found highly variable: from positive (melanoma) to neutral (renal cancer) to negative (breast cancer and CRC) correlations with patient outcome (reviewed in [21]). In fact, a very recent bioinformatics study across ~10,000 cancer biopsies from 50 types of hematological and solid malignancies described considerable inter-individual variation of Vγ9Vδ2 tumour-infiltrating lymphocyte (TIL) abundance, which reflected in variable associations with outcome [22].

While many factors can underlie the inter-individual variation of Vγ9Vδ2 T cell activities, we considered based on recent studies[23-27] that metabolic resources, namely lipids such as cholesterol, could affect Vγ9Vδ2 T cell activation and functions. Cholesterol is an essential component of membranes where it plays a major part in the regulation of membrane fluidity, and as such affects the activation of different receptor-mediated signal transduction pathways. In previous studies, it was shown that inhibition or genetic deletion of cholesterol esterification in CD8 T cells increases the cholesterol levels in the plasma membrane and enhances TCR signalling, promoting cytotoxic functions and proliferation[28]. Morover, genetic interference with cholesterol efflux via the ABCG1 transporter converted "pro-tumour" M2 into anti-tumour M1 macrophages and strongly suppressed tumour growth [29] Immune cell functions can thus clearly be affected by alterations in cholesterol homeostasis [25]. On the other hand, systemic cholesterol favours breast cancer progression by directly promoting breast cancer cells proliferation, migration and survival[30]. The predictive clinical value of elevated LDL-cholesterol levels in breast cancer patients at diagnosis is highly significant; women with elevated LDL-cholesterol levels at diagnosis have a significant higher risk of developing local recurrence or metastasis.

In this study we asked whether LDL impacts on Vγ9Vδ2 T cell activation and targeting of breast cancer cells. We found that activated Vγ9Vδ2 T cells express

LDL-R through which they uptake LDL, and this results in metabolic changes associated with reduced functionality, namely impaired IFN-γ production and reduced killing of breast cancer cells both in vitro and in vivo. This work thus demonstrates, for the first time, that LDL levels are important modulators of Vγ9Vδ2 T cell activation and anti-tumour functions.

2.3 Material and methods

2.3.1 Cell culture and in vitro killing assays

For Vγ9Vδ2 T cell culture and expansion, peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll- Hystopaque-1077; Sigma-Aldrich) for 30 minutes at 1,500 rpm and 25°C. Vγ9Vδ2 T cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 µg/mL of penicillin/streptomycin (Invitrogen, Gibco), in the presence of interleukin-2 (IL-2; Peprotech) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP; Echelon Biosciences). Fresh medium was added every 5 days, until day 14 of culture. Expanded Vγ9Vδ2 T cell populations were cultured for 36-72 hours at 37ºC and 5% CO2 in the presence or in the absence of low density human lipoproteins (LDL; 100µg/mL, Millipore), and tested for their anti-tumour activity. Cells were counted in Neubauer chamber using 0.4% Trypan Blue solution (Sigma-Aldrich) for viability control.

For tumour cell cultures and in vitro killing assays, the human breast cancer cell line MDA-MB-231 (ATCC) was cultured in DMEM medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Life Technologies); and in vitro-expanded Vγ9Vδ2 T cells were seeded in 96 well round-bottom plates. Tumour cells were stained with CellTrace Far Red DDAO-SE (1 µM; Molecular Probes; Invitrogen) and incubated at the indicated target: effector ratio with Vγ9Vδ2 T cells for 3 hours at 37ºC and 5% CO2. Cells were then stained with AnnexinV-FITC (BD Pharmingen) and analysed by flow cytometry.

For receptor blocking, cultured Vγ9Vδ2 T cells were pre-incubated for 1 hour with saturating amounts of blocking antibodies; human anti-TCR-γδ (clone B1), human

anti-NKG2D (clone 1D11), human anti DNAM-1 (11A8), human anti-FasL (clone NOK-1), human anti-CD2 (clone RPA-2.10), human-anti 2B4 (C1.7), mouse IG3k (clone MG3-35), all from Biolegend. Blocking antibodies were maintained in the culture medium during the killing assays.

2.3.2 Flow cytometry analysis

For cell surface protein staining, cells were labeled with fluorescent monoclonal antibodies: anti-CD3 (clone UCHT1, Biolegend), anti- CD45 (HI30, Biolegend), anti-CD69 (FN50, Biolegend), anti-TcRVγ9 (clone B3, Biolegend), anti-TcRVδ2 (clone B6, Biolegend), anti-LDL-R (FAB2148A, R&D Systems), anti-NKG2D (clone 1D11, Biolegend), anti-DNAM-1 (clone 11A8, Biolegend), anti-CD56 (clone HCD56, Biolegend), anti- CD279 (PD-1) NAT105, Biolegend). In all cultures, the percentage of Vγ9+ T-cells was evaluated by flow cytometry in a LSR Fortessa (BD Biosciences) flow cytometer.

For lipid droplet determination, expanded Vγ9Vδ2 T cells were stained with 0.5–1.5 μg/mL Nile Red (Sigma-Aldrich), followed by 10 min incubation in the dark at RT and analysis in a a LSR Fortessa flow cytometer. Alternatively, Vγ9Vδ2 T cells were pre-incubated for 10 min with 3.8 mM Bodipy (Invitrogen) at RT, washed twice with PBS followed by similar type of analyses (LSR Fortessa flow cytometer). For ROS quantification, cultured Vγ9Vδ2 T cells were stained with 1–10 μM ROS-CH-H2DFDA (ThermoFisher Scientific) fluorescent dyes for 30 min at 37ºC, washed (2X) and followed by similar type of analyses (BD LSR Fortessa flow cytometer). For determination of mitochondrial mass, cultured Vγ9Vδ2 T cells were washed with PBS and pre-incubated for 15 min with 2 nM MitoTracker Deep Red (Molecular Probes) at RT, after PBS wash, cells were analysed in LSR Fortessa flow cytometer. For cell proliferation, cultured Vγ9Vδ2 T cells were stained with CFSE (CellTrace CFSE Cell Proliferation Kit, Invitrogen) at 0.5 μM. Cell death in vitro was assessed by AnnexinV-FITC (BD Pharmingen) staining, where cultured Vγ9Vδ2 T cells were washed in PBS and resuspended in 300 μl AnnexinV binding buffer (BD Biosciences), containing AnnexinV-FITC (BD Pharmingen) and incubated for 15 minutes at RT before analysis in a LSR Fortessa flow cytometer. For cytokine detection, cultured Vγ9Vδ2 T cells were fixed in 25 ng/mL PMA

(Sigma Aldrich, P-8138), 2µg/mL Ionomycin (Sigma, I-0634) and 2µg/mL Brefeldin-A (Sigma, B-7651) in RPMI medium for 4 hours at 37ºC. For cell surface staining, Vγ9Vδ2 T cells were washed and stained with fluorescent antibodies for 10 min at 4ºC. For intracellular staining, cells were then resuspended in fix/perm buffer (BD Biosciences) and incubated for 30 min at 4ºC, followed by incubation in perm buffer with Fc-block (1:100) for 15 min. Antibodies for intracellular staining were added (1:100) for 30 min at 37ºC and 5% CO2. Concentrations of IFN-γ (B27), TNF-α (Mab-11) and IL-17A (BL-168) were assayed in a LSR Fortessa cytometer by flow (BD Biosciences).

2.3.3 Quantitative Real-Time PCR

RNA was extracted from cultured Vγ9Vδ2 T cells using TRIzol reagent (Invitrogen) according to the manufacturer protocol. Concentration was determined by spectrophotometry (Nanodrop 1000) and total RNA was reverse-transcribed into cDNA using random hexamers and Superscript II enzyme (Invitrogen). Quantitative real-time PCR (qPCR) was performed using SybrGreen reagent (Bio-Rad) in a ViiA 7 System sequence detection system (Applied Biosystems). Quantifications were made applying the ΔCt method (Ct of gene of interest-Ct of housekeeping gene) followed by 2^(-ΔΔCt). The housekeeping gene used for input normalization was β-2 microglobulin. Primers used in the quantitative PCR assays were: LDL-R, Fwd: 5´-GCTTGTCTGTCACCTGCAAA-3´; LDL-R, Rev: 5´-AACTGCCGAGAGATGCACTT-3´; CD69, Fwd: 5´-CAAGTTCCTGTCCTGTGTGC-3´; CD69, Rev: 5´-GAGAATGTGTATTGGCCTGGA-3´; IFN-g, Fwd: 5` GGCATTTTGAAGAATTGGAAAG-3´; IFN-g, Rev: 5´- TTTGGATGCTCTGGTCATCTT-3´; FASL, Fwd: 5-´GTTCTGGTTGCCTTGGTAGG-3´; FASL, Rev: 5`-TGTGCATCTGGCTGGTAGAC-3´; STAT1, FW: 5´-AGTTCGGCAGCAGCTTAAAA-3`; STAT1, Rver: 5-´TGTCTTTCCACCACAAACGA-3´.

For mtDNA determination, expanded Vγ9Vδ2 T-cells (1 × 10^6 cells/mL) were ressuspended with protein K and incubated at 65ºC for 3 hours with vertical rotation. Total DNA was isolated from cells using phenol:chloroform:isoamyl alcohol (Sigma) and measured by assessing the levels of the human mitochondrial ND1 (human mtND1: 5'-CCCTAAAACCCGCCACATCT-3' and 5'-

GAGCGATGGTGAGAGCTAAGGT-3') relative to nuclear β2-microglobulin gene (5'-TCGCTCCGTGGCCTTAGCTGT-3' and 5'-CTTTGGAGTACGCTGGATAGCCTCC-3') using SybrGreen reagent (Bio-Rad) and ViiA 7 System sequence detection system (Applied Biosystems).Quantifications were made applying the ΔCt method (Ct of nuclear DNA gene - Ct of mitochondrial DNA gene) followed by 2x2^(ΔCt) according to others (31).

2.3.4 Histological analysis

For detection of lipid droplets, poly-lysine was used to coat slides and promote attachment of Vγ9Vδ2 T cells. Then, Vγ9Vδ2 T cells were fixed with 4% paraformaldehyde in PBS for 15 min and stained with 0.1 μg/mL Bodipy (Invitrogen) in PBS, at RT for 15 min. Cells were washed three times with PBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame). Images were captured using a Zeiss LSM710 confocal microscope.

The immunohistological stainings for Ki67 (MIB-1) and human CD3 in subcutaneous tumours were performed in 4μm sections following conventional protocols. Briefly, for antigen retrieval, the slides were treated in a PT Link module (DAKO) at low-Ph, followed by incubation with the primary antibodies. EnVision Link horseradish peroxidase/DAB visualization system (DAKO) was used and counterstained with Harris haematoxylin. Slides were scanned in the Hamamatsu NanoZoomerSQ.

2.3.5 In vivo tumour experiments

All animal experiments were performed in accordance with national guidelines from the "Direção Geral de Veterinária" and approved by the national Ethics Committee. NSG mice were obtained from Jackson Laboratories. Age 5–7 week old female mice were injected with MDA-MB-231 Luciferase-GFP (pre-transduced with a lentiviral vector encoding luciferase and GFP and enriched for GFP+ cells, data not shown) breast cancer cells in the right axillary mammary fat pad and,

after tumour was established (day 17), Vγ9Vδ2 T cells or PBS (control) were injected twice. After 19 days of treatment, animals were sacrificed and organ, tumour and blood were collected for further analysis.

2.3.6 Statistical analysis

In vitro and *in vivo* data are presented as the mean± s.d. Statistical analyses were performed using Student t test or ANOVA. Unpaired Student's t-test and one-way analysis of variance were used for comparison of experimental groups. Statistical analysis was performed using GraphPad Prism software version 6.

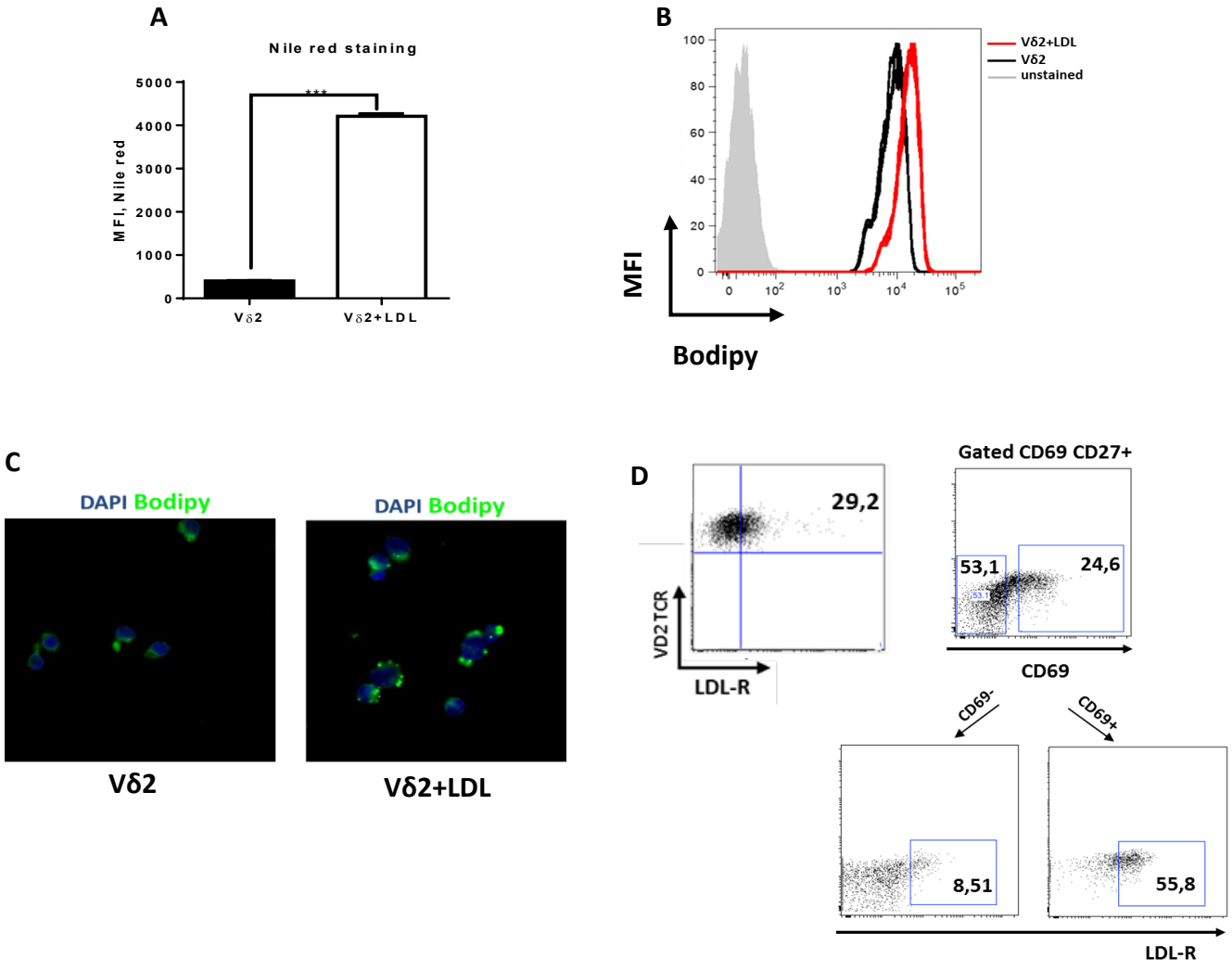
2.4 Results

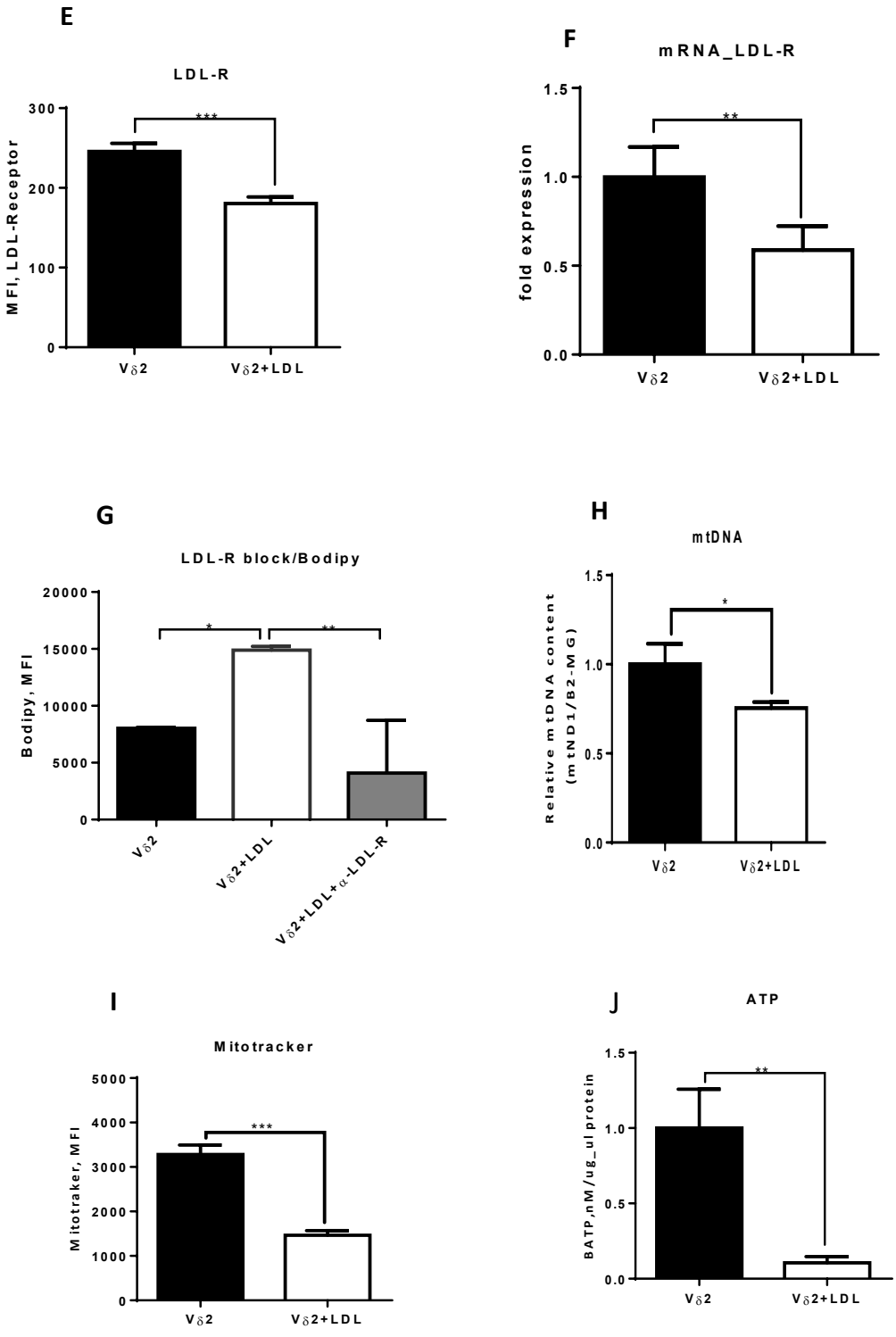
2.4.1 Activated Vγ9Vδ2 T cells uptake LDL-cholesterol via LDL-Receptor

We started this study by investigating the capacity of Vγ9Vδ2 T cells to sense, i.e. uptake, native LDL. For this purpose, we activated and expanded γδ T cells in vitro up to 14 days with IL-2 plus the most potent phosphoantigen, hydroxyl-methyl-butinyl pyrophosphate (HMB-PP), which resulted in ~80% Vδ2+ T cells (Fig. S1A). This is the type of cellular product being used in adoptive cell transfer approaches[19], which highlights its therapeutic relevance. Importantly, in all our flow cytometry analyses we specifically gated on Vδ2+ T cells, to avoid interference of potential "contaminants" in the cellular product. We will thus refer herein to this phosphoantigen-activated and Vγ9Vδ2 T cell-enriched cellular product simply as "activated Vγ9Vδ2 T cells". We exposed them for 36-72h in the presence of LDL (in the same culture medium used for the expansion), and used Bodipy 493/503 and Nile Red to clearly document the internalization and intracellular accumulation of cholesterol (Fig. 2.4.1A-C). This was observed at 100µg/mL but not at 50µg/mL LDL (data not shown), and thus we used the former concentration for all subsequent experiments. Importantly, whereas ex vivo Vδ2+ (or Vδ1+) T cells rarely expressed the receptor (Fig. S1B), including in the tumour environment (Fig. S1C), Vγ9Vδ2 T cells expressed (~30%) LDL-R upon activation (Fig.2.4.1D and Fig. S1D). The association of LDL-R expression with Vγ9Vδ2 T cell activation was further documented by its co-segregation with the activation marker CD69 (Fig. 2.4.1D). Of note, non-Vδ2+ cells within the cultures, and in particular Vδ1+ T cells, failed to increase their lipid content (Fig. S1E), which thus restricts LDL uptake to activated Vγ9Vδ2 T cells.

Moreover, the exposure of activated Vγ9Vδ2 T cells to LDL led to a downregulation of LDL-R expression (Fig. 2.4.1E), suggestive of receptor internalization; and LDL-R blockade with specific monoclonal antibodies fully inhibited the accumulation of cholesterol in Vγ9Vδ2 T cells (Fig. 2.4.1F). These data clearly demonstrate that LDL-R expression by activated Vγ9Vδ2 T cells endows them with the capacity to uptake LDL.

We next characterized the effects of LDL exposure and uptake on metabolic features of Vγ9Vδ2 T cells. We observed a significant reduction in mitochondrial mass (Fig. 2.4.1G), mitochondrial DNA content (Fig. 2.4.1H) and production of reactive oxygen species (Fig. 2.4.1I), which was accompanied by severely decreased cellular ATP levels (Fig. 2.4.1J) in Vγ9Vδ2 T cells exposed to LDL. Importantly, the effects of LDL were not accompanied by a decrease in cell viability, as demonstrated by quantification of apoptosis of Vγ9Vδ2 T cells exposed (or not) to LDL (Fig. S1F). Thus, LDL uptake has a substantial impact on the metabolic output of Vγ9Vδ2 T cells, which questions changes in their functionality.




E
LDL-R
MFI, LDL-Receptor
300
200
100
0
***
Vδ2
Vδ2+LDL
F
mRNA_LDL-R
fold expression
1.5
1.0
0.5
0.0
**
Vδ2
Vδ2+LDL
G
LDL-R block/Bodipy
Bodipy, MFI
20000
15000
10000
5000
0
*
**
Vδ2
Vδ2+LDL
Vδ2+LDL+α-LDL-R
H
mtDNA
Relative mtDNA content (mtND1/B2-MG)
1.5
1.0
0.5
0.0
*
Vδ2
Vδ2+LDL
I
Mitotracker
Mitotraker, MFI
5000
4000
3000
2000
1000
0
***
Vδ2
Vδ2+LDL
J
ATP
BATP,nM/ug_ul protein
1.5
1.0
0.5
0.0
**
Vδ2
Vδ2+LDL

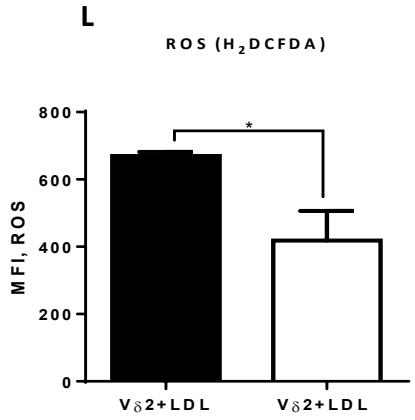


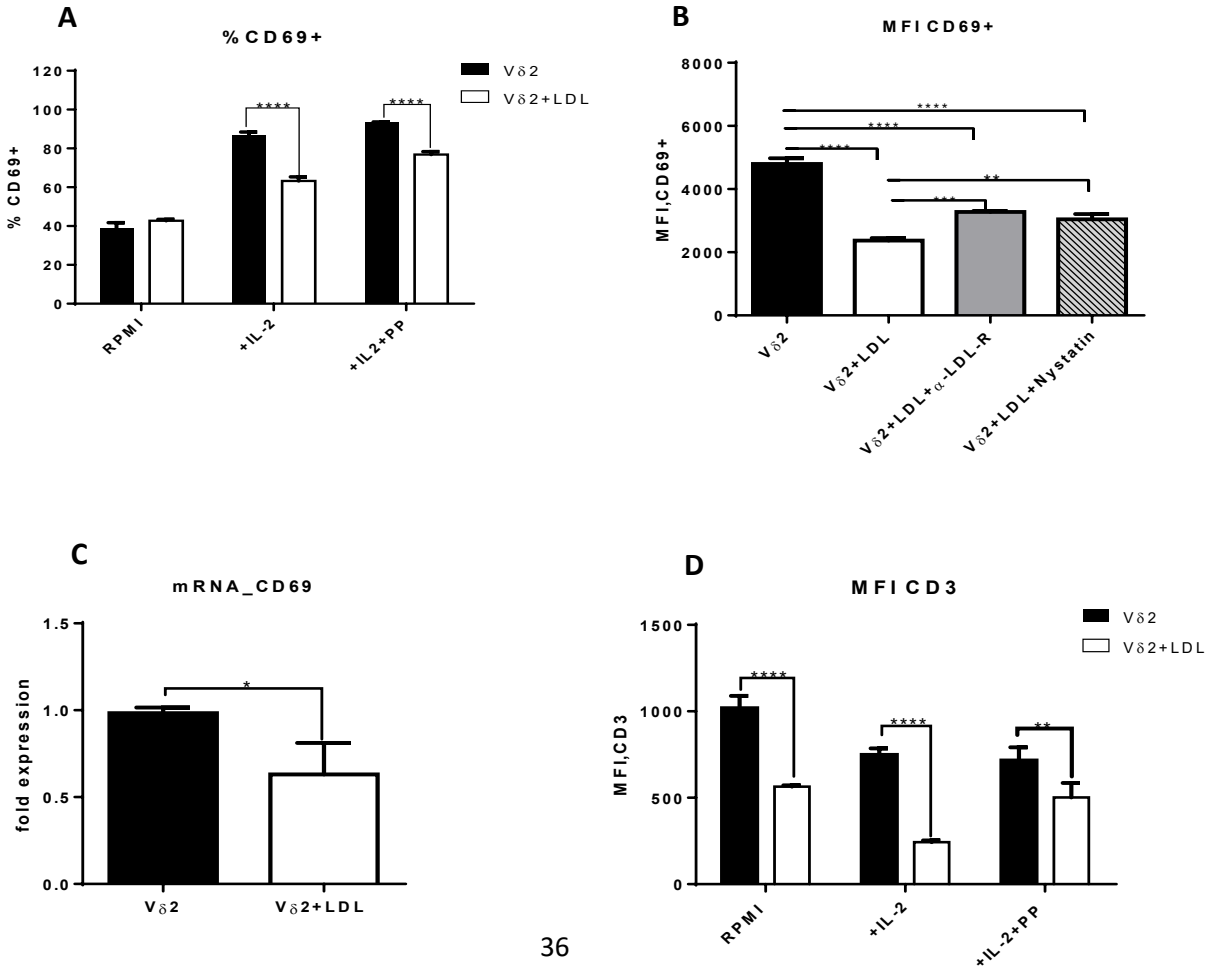
Figure 2.4.1: **Activated Vγ9Vδ2 T cells uptake LDL-cholesterol via LDL-Receptor**

Activated and expanded γδ (~80% Vδ2$^+$) T cells were cultured in RPMI 1640 medium with IL-2 and HMB-PP ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate) in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours. LDL-Cholesterol uptake was assessed by Nile red (A) and Bodipy (B, C) lipid droplet staining by flow cytometry. (D) Flow cytometry plots for control (in the absence of LDL treatment) LDL-receptor (LDL-R) expression in gated Vδ2$^+$ T cells (*left*); or segregated on the basis of CD69 expression (*right*). (E) Mean fluorescence intensity (MFI) for LDL-R expression on Vδ2$^+$ T cells after 72 hours of incubation in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL). (F) RT-qPCR analysis of the mRNA expression of *LDL-R*, normalized to the housekeeping gene *β2-microglobulin*. (G) MFI for Bodipy lipid droplet staining in expanded Vδ2$^+$ T cells, cultured in the presence of LDL only or in the presence of an anti-LDL-R antibody. (H-J) Mitochondria mass measured by MitoTracker Deep Red staining (H), relative mitochondrial DNA (mtDNA) content determined by qPCR quantification in DNA samples (I), ATP production (J) and reactive oxygen species (ROS) content by H_2DCFDA staining (L) in cell extracts obtained as in (E). Data are from three independent experiments and are presented as mean ± s.d. *P<0.05,**P<0.01 and ***P<0.001.

2.4.2 LDL-cholesterol inhibits Vγ9Vδ2 T cell activation and cytokine production

Activated Vγ9Vδ2 T cells express the surface marker CD69 and produce cytotoxic and pro-inflammatory cytokines (5-7). Here, we tested whether these functional properties were affected by LDL uptake over a 72h period in which pre-expanded/ activated Vγ9Vδ2 T cells were incubated with medium alone, IL-2 or IL-2 + HMB-

PP. We observed a consistent decrease in the proportion of CD69^{+} cells (Fig. 2.4.2A and Fig. S2), as well as CD69 protein (Fig. 2.4.2B) and mRNA (Fig. 2.4.2C) expression levels in Vγ9Vδ2 T cells upon exposure to LDL. The impairment in CD69 expression was partially reverted by LDL-R blockade or nystatin (cholesterol sequestering agent) treatment (Fig. 2.4.2B), which sequesters cellular cholesterol when used *in vitro* assays. The impact on Vγ9Vδ2 T cell activation was further documented by the significant downregulation of CD3 expression (Fig. 2.4.2D). Most importantly, LDL uptake strongly affected IFN-γ expression at both protein (Fig. 2.4.2E) and mRNA (Fig. 2.4.2F) levels, the latter associating with a downregulation of the transcriptional regulator of Ifng expression, Stat-1 (Fig. 2.4.2G). Of note, other functional properties, such as TNFα or IL-17 production, were not affected by LDL exposure (Fig. S3). These data demonstrate that LDL uptake limits Vγ9Vδ2 T cell activation and their capacity to produce the signature anti-tumour cytokine, IFN-γ. We next assessed its impact on anti-tumour cytotoxicity.



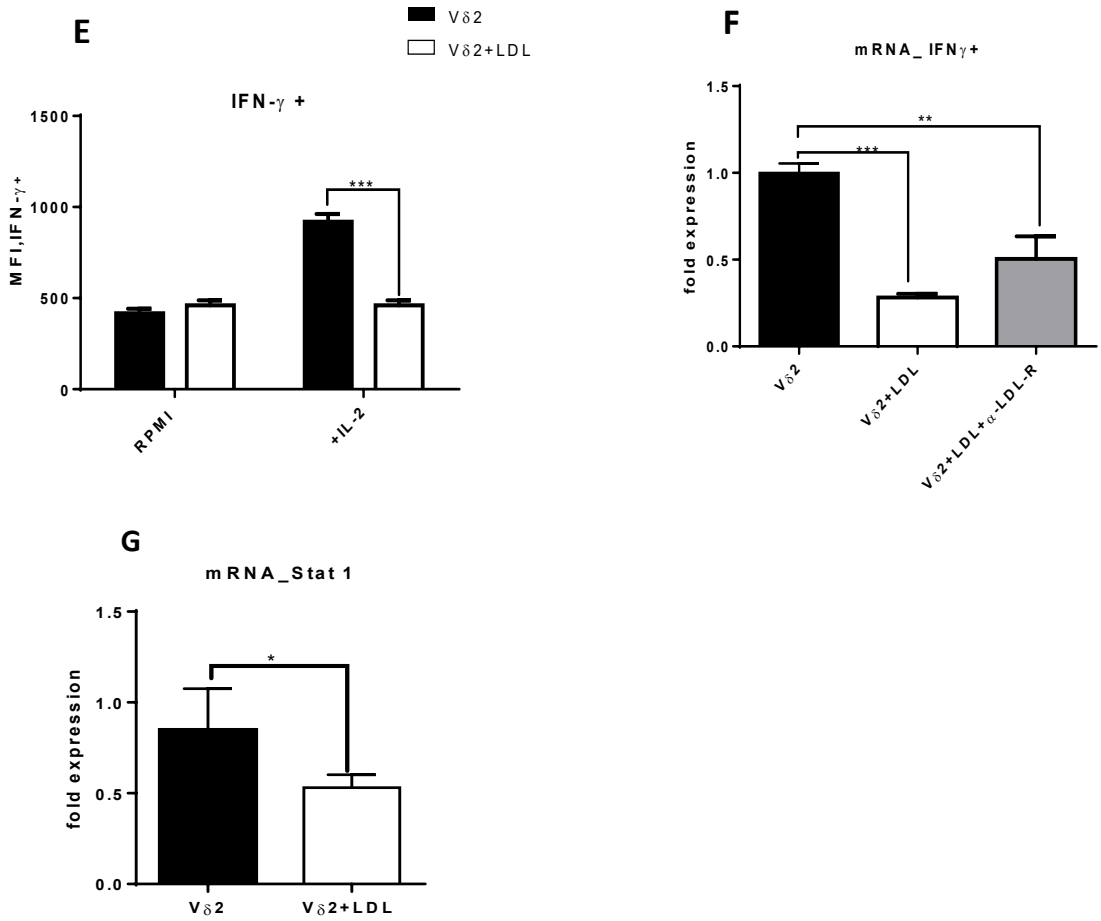
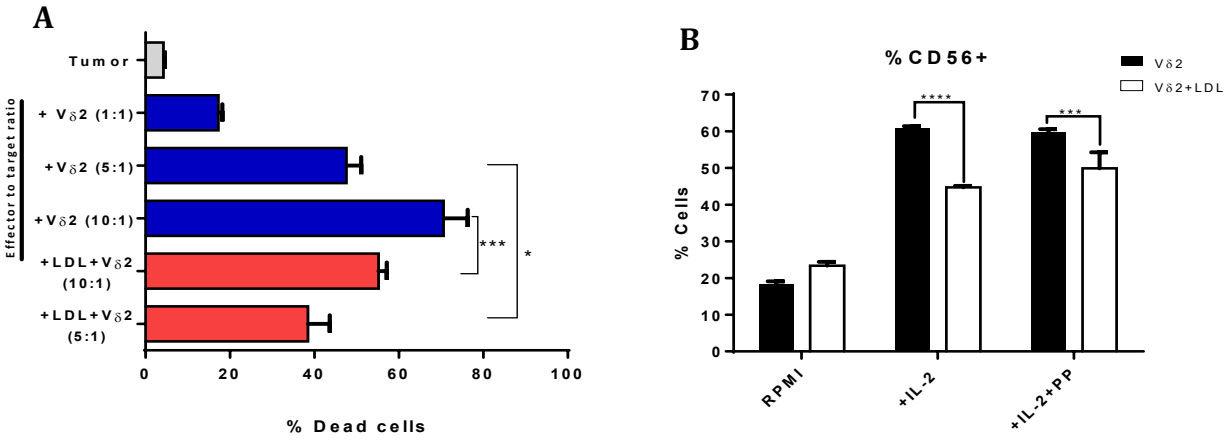


Figure 2.4.2: LDL-cholesterol inhibits Vγ9Vδ2 T cell activation and cytokine production

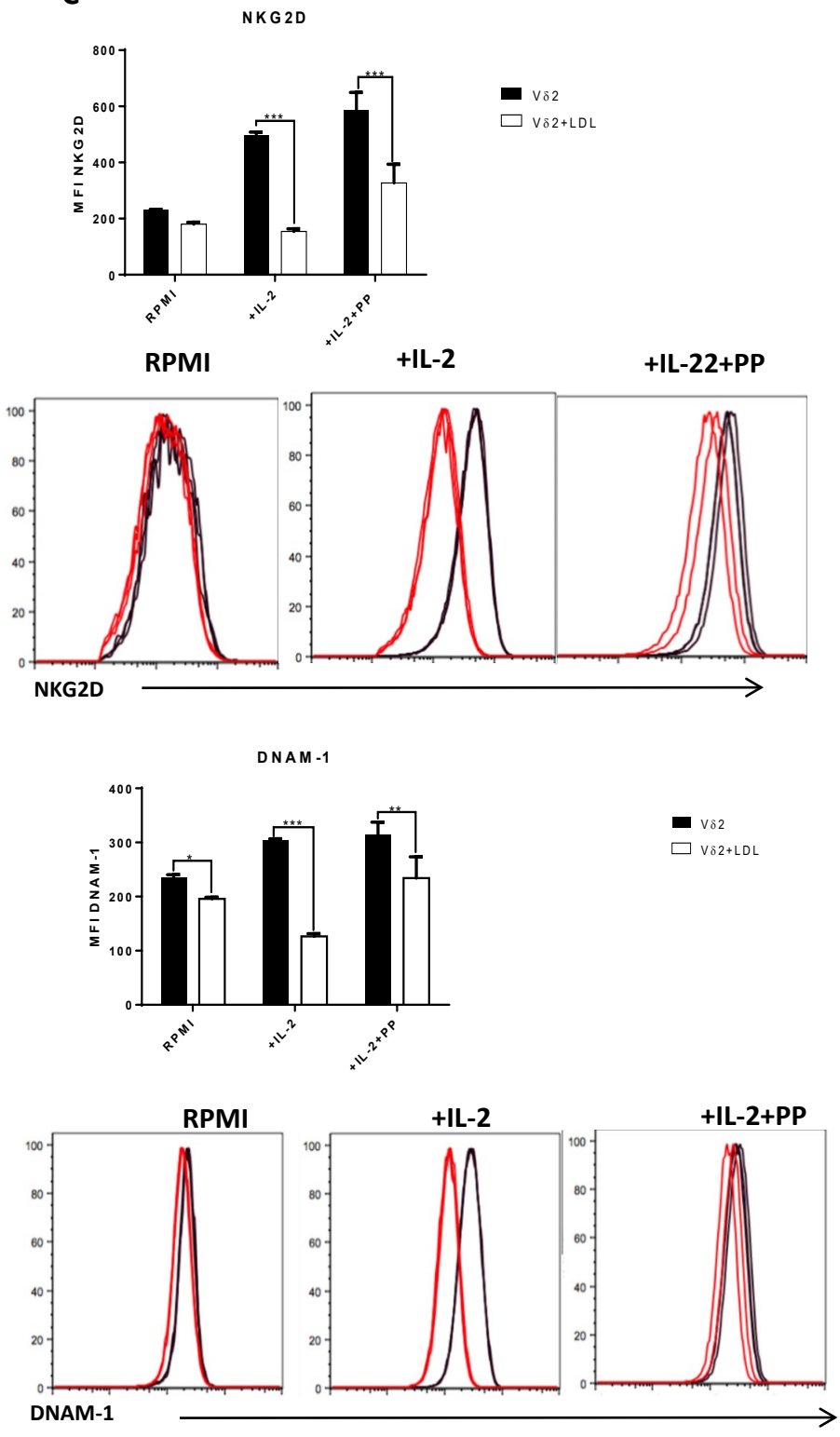
Pre-expanded γδ (~80% Vδ2+) T cells were cultured in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours. (A-B) Percentage of CD69+ Vγ9Vδ2 T cells (A) and MFI for CD69 expression (B), in the absence or presence of LDL and, when indicated, anti-LDL-R antibody or Nystatin. (C) RT-qPCR analysis of the mRNA expression of CD69, normalized to the housekeeping gene β2-microglobulin. (D) MFI for CD3 surface expression. (E) MFI for intracellular IFN-γ expression. (F-G) RT-qPCR analysis of the mRNA expression of IFN-γ (F) and STAT-1 (G) normalized to the housekeeping gene β2-microglobulin. Data are from three independent experiments and are presented as mean ± s.d. *P<0.05,**P<0.01 and ***P<0.001, 2-tailed Student t test.

2.4.3 LDL-cholesterol downregulates NKG2D and DNAM-1 and reduces γδ T cell cytotoxicity in vitro

Activated Vγ9Vδ2 T cells killed the breast cancer cell line, MDA-MB-231, in a dose-dependent manner; importantly, exposure to LDL consistently inhibited this cytotoxic function (Fig. 2.4.3A). This associated with reduced expression of the cytotoxicity-associated marker, CD56, in pre-expanded/ activated Vγ9Vδ2 T cells kept on IL-2 alone or HMB-PP plus IL-2 (Fig. 2.4.3B). To gain further mechanistic insight, we assessed two key determinants of anti-tumour Vγ9Vδ2 T cell cytotoxicity, NKG2D[16] and DNAM-1[17]. The exposure to LDL significantly compromised the expression of both receptors, both in the presence of serum (Fig.2.4. 3C and Fig. S2) or human plasma (Fig. S4), which were required for MDA-MB-231 cell targeting by pre-activated Vγ9Vδ2 T cells, as demonstrated by antibody blockade during the *in vitro* killing assay (Fig. 2.4.3D). Of note, NKG2D and DNAM-1 played synergistic roles in tumour cell recognition, since the blockade of each individual receptor had negligible effects on the killing assay (Fig. S5). In addition, LDL exposure prevented any impact of NKG2D/ DNAM-1 blockade, further supporting the role of these receptors in the cytotoxic mechanisms impaired in Vγ9Vδ2 T cells (Fig. 2.4.3E). These data extended the impact of LDL-cholesterol uptake to anti-tumour cytotoxicity, which prompted to test the functionality of LDL-exposed Vγ9Vδ2 T cells in an in vivo breast cancer model.



C


NKG2D
MFI NKG2D
800
600
400
200
0
***
***
RPMI
+IL-2
+IL-2+PP
Vδ2
Vδ2+LDL
RPMI
+IL-2
+IL-22+PP
100
80
60
40
20
0
NKG2D
DNAM-1
MFI DNAM-1
400
300
200
100
0
*
***
**
RPMI
+IL-2
+IL-2+PP
Vδ2
Vδ2+LDL
RPMI
+IL-2
+IL-2+PP
DNAM-1

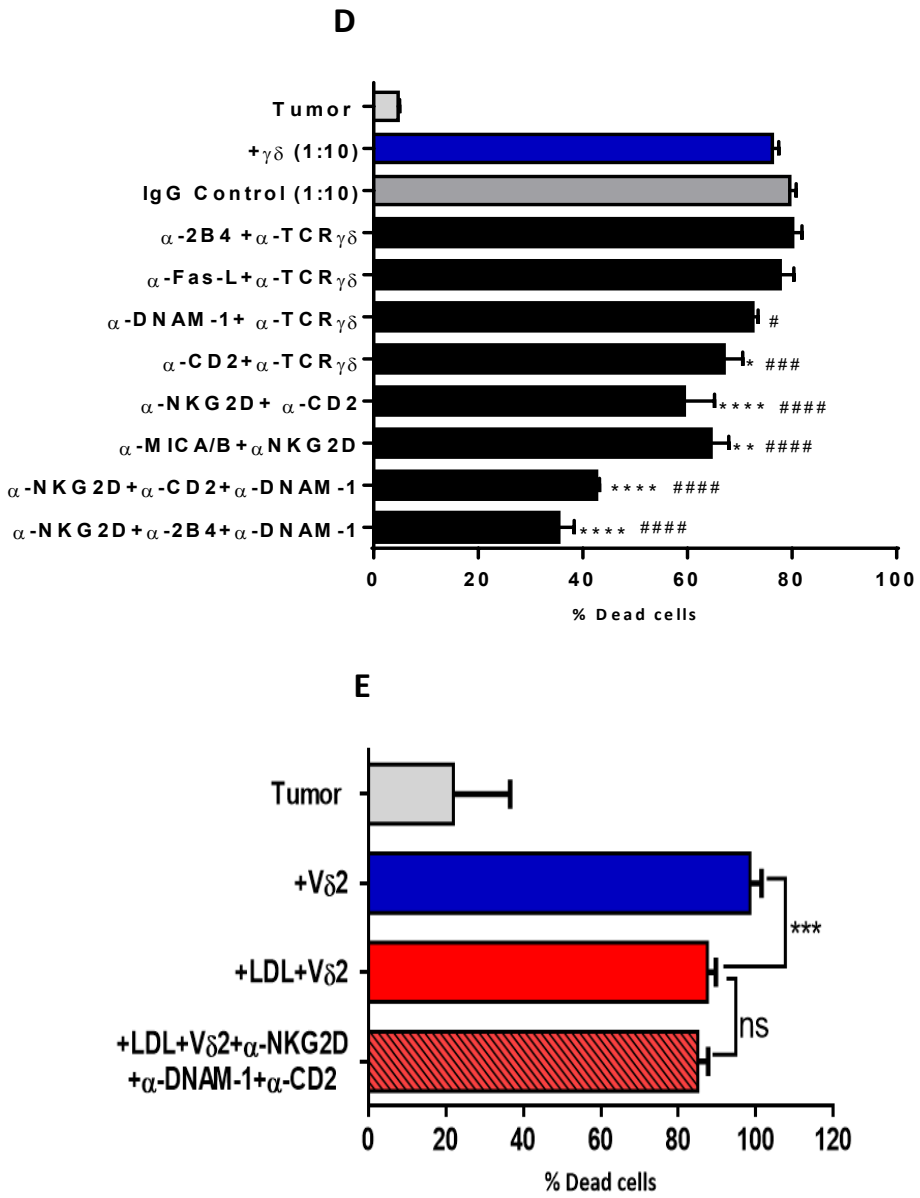
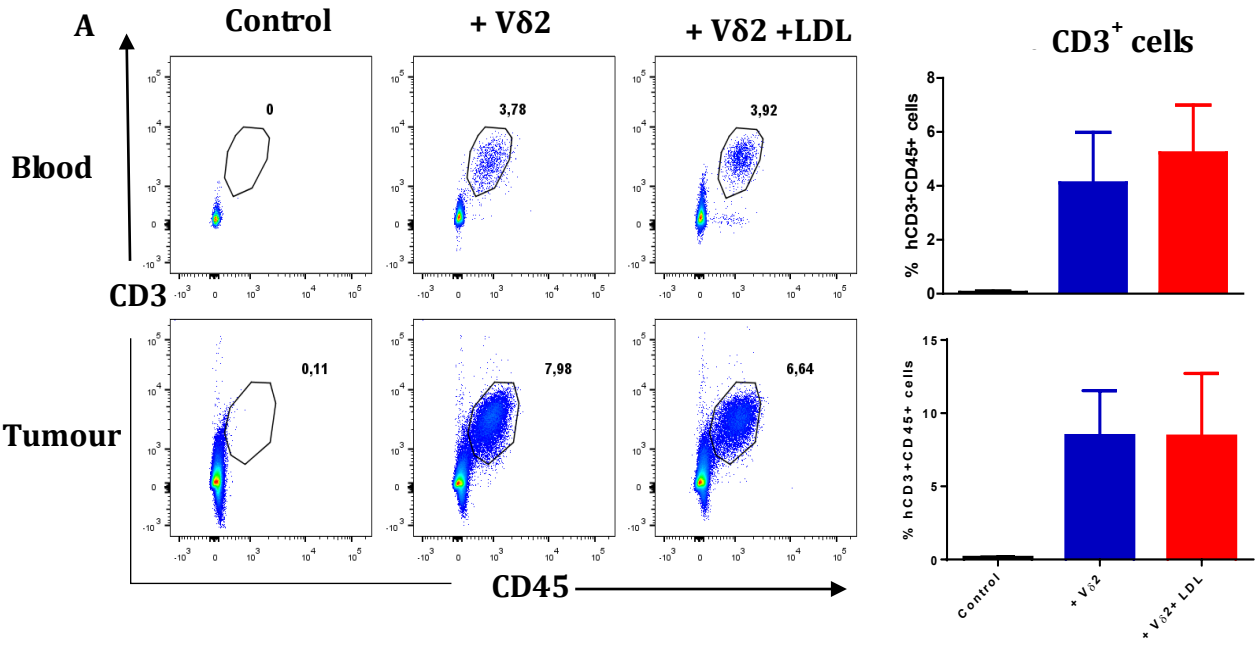


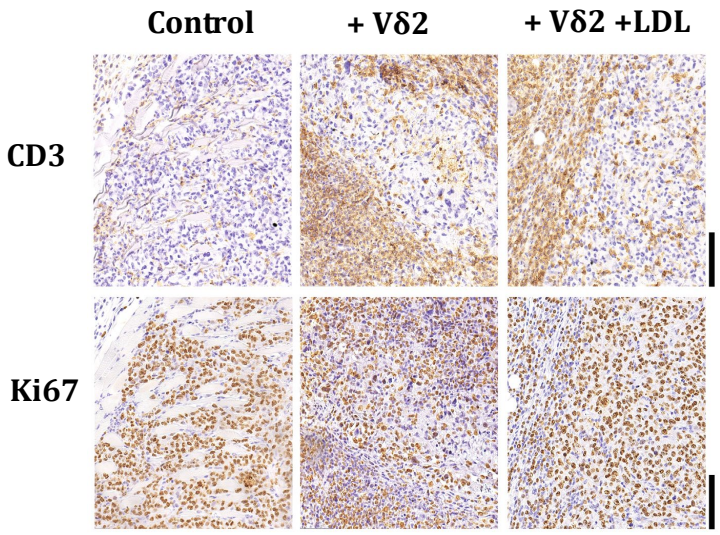
Figure 2.4.3: Pre-expanded γδ (~80% Vδ2+) T cells were cultured in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours. (A) In vitro killing assays upon co-culture for 3 hours with the human breast cancer cell line MDA-MB-231 (at 1:1, 5:1, 10:1 effector: target ratios). The death of target cells (pre-labeled with DDAO-SE dye) was assessed by Annexin-V staining and flow cytometry. (B) Percentage of CD56 surface expression. (C) Expression of the cytotoxicity receptors, NKG2D (upper panel) and DNAM-1 (lower panel) determined by flow cytometry, depicted as representative histograms (left) and quantification (MFI, right). (D) Effect of combinations of blocking antibodies against surface receptors in in vitro killing assays with Vγ9Vδ2 T cells co-cultured for 3 hours with MDA-MB-231 breast cancer cells at 10:1 effector: target. (E) Effect of the combination of blocking antibodies against NKG2D, DNAM-1 and CD2 on LDL-exposed Vγ9Vδ2 T cells co-cultured with MDA-MB-231 at 10:1 effector: target ratio. The death of target cells (pre-labeled with DDAO-SE dye) was assessed by Annexin-V staining and flow cytometry. Data are representative of three independent experiments and are presented as mean ± s.d. * and # indicate significant differences relative to IgG isotype control or γδ, respectively. *, # P<0.05, **, ##, P<0.01 and ***, ### P<0.001.

2.4.4 LDL-cholesterol limits the anti-tumour therapeutic effect of human γδ T cells in vivo

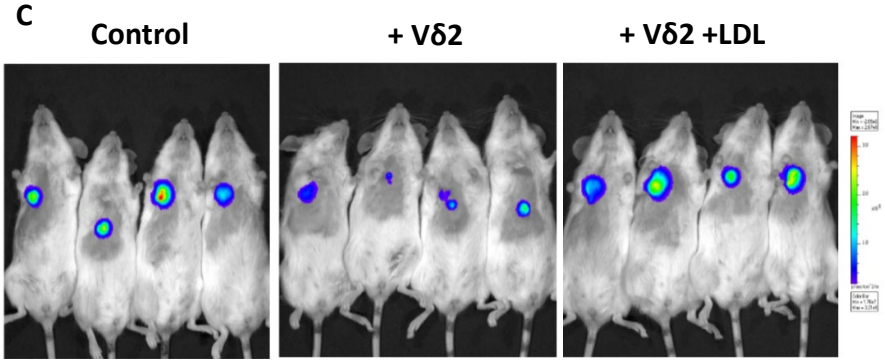
We used immunodeficient mice (NSG) injected with the human breast cancer cell line MDA-MB-231luc+ (expressing luciferase) to test the therapeutic potential of adoptive transferring Vγ9Vδ2 T cells (in 2 injections) exposed or not to LDL. First, we confirmed that the human ($CD3^+$) T cells could be detected in the blood of inoculated mice (Fig. 2.4.4A), and readily infiltrated the tumours, as determined by flow cytometry (Fig. 2.4.4A) and immunohistochemistry (Fig. 2.4.4B). LDL treatment did not affect human T cell abundance in either the blood or within the tumour, showing that LDL exposure does not impact on their implantation, expansion or migration capacities (Fig. 2.4.4A-B). However, the LDL-exposed group showed a dramatic impairment in controlling tumour growth when compared to "LDL-naïve" Vγ9Vδ2 T cells (Fig. 2.4.4C-D). In fact, LDL-exposed Vγ9Vδ2 T cells were unable to have any therapeutic impact (as compared to the PBS-injected control group) (Fig. 2.4.4C-D). These data clearly demonstrate that LDL uptake has a long-lasting negative impact on Vγ9Vδ2 T cell activation, which results in a severe impairment of their anti-tumour functions *in vivo*.

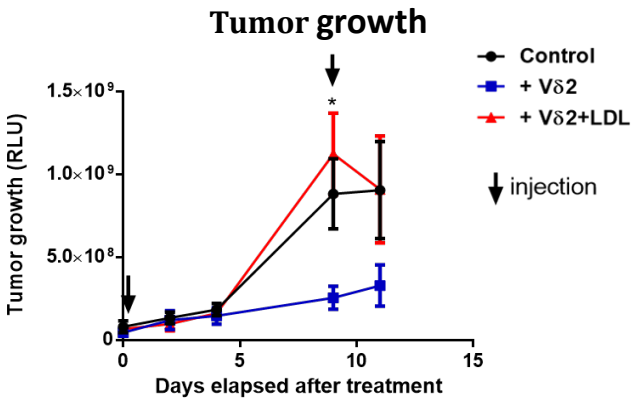


B


Control
+ Vδ2
+ Vδ2 +LDL
CD3
Ki67

C


Control
+ Vδ2
+ Vδ2 +LDL


Tumor growth
Control
+ Vδ2
+ Vδ2+LDL
injection
Tumor growth (RLU)
1.5×10⁹
1.0×10⁹
5.0×10⁸
0
0
5
10
15
Days elapsed after treatment
*

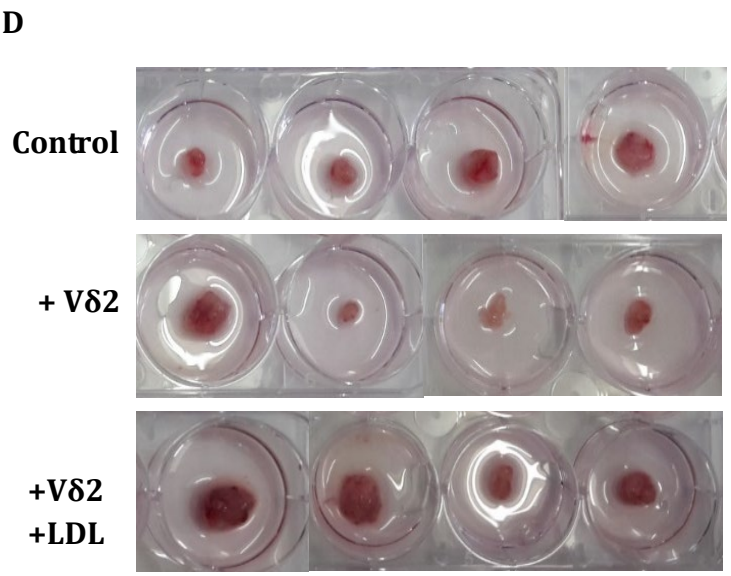


Figure 2.4.4: LDL-cholesterol limits the anti-tumour therapeutic effect of human γδ T cells in vivo

NSG mice were injected with 1x10^6 human breast cancer MDA-MB-231 luciferase+ cells and, after tumour was fully established (day 17), pre-expanded Vδ2 T cells untreated (Vδ2) or treated with LDL-cholesterol (Vδ2+LDL), or PBS (as control), were injected twice. At the end of the experiment, animals were sacrificed and organ, tumour and blood were collected for analysis. (A) Flow cytometry analysis of T cells (CD45+ CD3+) in blood and tumour mass in mice from the different experimental groups. (B) Immunohistochemistry microphotographs for CD3 and Ki67 in subcutaneous tumours; DAB counterstained with Harris hematoxylin. Original magnification 20x (bar 100μm). (C) MDA-MB-231 tumour bearing NSG mice (n=4 per group) were analyzed by IVIS Lumina imager. Representative pictures of bioluminescence imaging at terminus and temporal evaluation/quantification along the experiment. (D) Representative images of the tumours dissected at the end of the experiment. Data are presented as mean ± s.d. *P<0.05, **P<0.01 and ***P<0.001.

2.5 Discussion

Altered lipid metabolism is being increasingly recognized as a hallmark of cancer[30]. This metabolic change is modulated by oncogenic signaling pathways and is important for the initiation and progression of tumours, since cellular growth is dependent on the sustained availability of lipids [33]. Moreover, metabolic shifts in lipid metabolism were shown to drive tumour recurrence after therapeutic intervention[34]. Among the frequent alterations in lipid metabolism, *de novo* lipid biosynthesis has been suggested to play a major role in cancer pathogenesis [35]. Moreover, there is emerging evidence that the uptake of exogenous lipids by tumour cells and by non-malignant cells in the tumour microenvironment may also contribute to malignancy [36-38]. Importantly, this particular metabolic feature has been pointed out as a possible explanation for the association of some cancers, including breast cancer, and high fat/high cholesterol diets [39].

Cholesterol is an essential component of cell membrane microdomains, including lipid rafts [40], and as such, it is essential for the activation of signal transduction pathways, intracellular trafficking, polarity and cell migration. We and others had previously shown that LDL-cholesterol favors breast cancer growth by directly modulating cancer cell properties[23,30]. On the other hand, genetic interference with cholesterol efflux (via the ABCG1 transporter) was found to convert "pro-tumour" M2 into anti-tumour M1 macrophages and strongly suppress tumour growth [41]. However, it remained to be addressed whether LDL-cholesterol had additional roles on tumour-infiltrating lymphocytes (TILs); or on cellular products to be used on adoptive cell therapy (ACT) of cancer. In the present study, we investigated whether LDL-cholesterol affected the activation and anti-tumour activity of human Vγ9Vδ2 T-cells, a promising ACT candidate. We found that LDL is internalized and accumulates in Vγ9Vδ2 T-cells, leading to reduce Vγ9Vδ2 T-cell activation, mitochondrial mass and ATP production, and greatly inhibiting their anti-tumour function *in vitro* and *in vivo* (xenograft model of human breast cancer).

Cholesterol metabolism seems to be an important mediator of T cell function and LDL-R is pivotal to cellular regulation. LDL-R downregulation upon exposure to LDL may be a mechanism by which Vγ9Vδ2 T-cells limit the intracellular accumulation of cholesterol, which has significant toxic effects[42]. Thus, when

Vγ9Vδ2 T cells exposed to LDL were treated with an LDL-R neutralizing antibody, or with Nystatin (a cholesterol-sequestering agent), their activation status was rescued. In contrast, a recent study in a mouse model of melanoma demonstrated that the inhibition of cholesterol esterification on $CD8^+$ tumour infiltrating T lymphocytes (TILs) by genetic ablation or pharmacological inhibition of ACAT1 (a key cholesterol esterification enzyme), led to increased levels of cholesterol in the plasma membrane, and potentiated the anti-tumour effector function and enhanced proliferation of $CD8^+$ T cells [28]. These data suggest that an "optimal" content of intracellular cholesterol is necessary to support T cell functions. Along these lines, free fatty acid uptake and usage by mitochondrial oxidative metabolism were recently shown to be critical for the long-term persistence of tissue-resident memory $CD8^+$ T cells (generated in response to viral infection) in the skin [43]. As such, the inhibitory effects of LDL-cholesterol on Vγ9Vδ2 T cells we unravel here may suggest a physiological threshold for intracellular lipid accumulation on T cells, although at this stage we cannot exclude that distinct T cell subsets may have different sensitivities and coping mechanisms.

The importance of metabolism on the regulation of immune cell function has been under intense scrutiny in recent years. Particularly in the case of TILs, it is known that if these cells cannot sustain mitochondrial function, their effector function is compromised. Accordingly, recent studies have suggested that rescuing mitochondrial biogenesis in effector T cells can augment their anti-tumour immunity [44,45]. In line with these studies, we showed here that Vγ9Vδ2 T-cells exposed to LDL-cholesterol have lower amounts of mitochondrial DNA and reduced mitochondrial content, which was accompanied by a decrease on ATP production. How a decrease in mitochondrial content and function affects the activation of effector T cells, including Vγ9Vδ2 T cells, is still to be established. Of relevance, recently it was reported no significant changes in the metabolism of $CD8^+$ T cells upon inhibition of ACAT1, even after an increase in intracellular cholesterol levels [28].

Vγ9Vδ2 T cells exposed to LDL-cholesterol also showed impaired production of IFN-γ, a key determinant of Vγ9Vδ2 T-cell anti-tumour responses [1,46]. We showed that LDL-cholesterol reduces the expression of Stat-1, which is known to regulate

the production of IFN-γ expression by γδ T cells [47]. Along this line, but by a different mechanism, cholesterol depletion was shown to increase Vγ9Vδ2 T cell cytotoxicity against PC-3 prostate cancer cells through the upregulation of the mevalonate pathway on target cells [48]. Furthermore, recent studies suggest that elevated cholesterol levels (hypercholesterolemia) induces downregulation of Tet1 in hematopoietic stem cells, leading to the inhibition of NKT and γδ T cell differentiation and increased colorectal cancer incidence[49]. In the present study we also found a significant reduction in multifunctional IFN-γ^+ TNF-α^+ γδ T cells infiltrating tumour lesions in the widely-used syngeneic E0771 model of breast cancer (Fig. S6).

Our work showed that the expression of cytotoxicity-associated receptors, namely NKG2D and DNAM-1, which determine tumour susceptibility to γδ T cell–mediated cytolysis, were strongly downregulated upon exposure of Vγ9Vδ2 T cells to LDL. This occurred in pre-expanded/ activated Vγ9Vδ2 T cells kept on either IL-2 alone or combined with HMB-PP, thus showing that phosphoantigen stimulation is not able to compensate the inhibitory effect on LDL on Vγ9Vδ2 T cell activation. Thus, LDL interferes with the two key stages of Vγ9Vδ2 T cell functionality[18] TCR-mediated activation (as indicated by impaired CD69 and IFN-γ production) and NK receptor-mediated tumour targeting (via down-regulation of NKG2D and DNAM-1). Importantly, we further demonstrated that the *in vitro* effects of LDL-cholesterol on Vγ9Vδ2 T-cell functions translated in the abrogation of their therapeutic effects *in situ* in the xenograft model of human breast cancer.

Taken together, our findings provide new evidence and contribute towards a better understanding on how tumours may evade immune surveillance in the context of hypercholesterolemia. Of note, recent studies suggest there is a high incidence of elevated LDL levels in Chronic Lymphocytic Leukemia (CLL) patients and a survival benefit from cholesterol-lowering statin drugs[50]. Prospective clinical trials are needed to confirm the therapeutic potential of lowering LDL concentrations in CLL and other cancer types. We further propose that upcoming research should specifically investigate the impact of (controlling) LDL levels on the outcome of the various cancer immunotherapy strategies being evaluated in the clinic.

2.6. References

1. Silva-Santos B, Serre K, Norell H. [gamma][delta] T cells in cancer. Nat Rev Immunol2015;15(11):683-91.

2. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol2010 Jul;10(7):467-78.

3. Lafont V, Sanchez F, Laprevotte E, Michaud HA, Gros L, Eliaou JF, et al. Plasticity of gammadelta T Cells: Impact on the Anti-Tumor Response. Front Immunol2014;5:622.

4. Casetti R, Martino A. The plasticity of gamma delta T cells: innate immunity, antigen presentation and new immunotherapy. Cell Mol Immunol2008 Jun;5(3):161-70.

5. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human [gamma][delta] T cells. Nature1995;375(6527):155-8.

6. Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, et al. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. Immunity1995 Oct;3(4):495-507.

7. Harly C, Guillaume Y, Nedellec S, Peigne CM, Monkkonen H, Monkkonen J, et al. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human gammadelta T-cell subset. Blood2012 Sep 13;120(11):2269-79.

8. Sandstrom A, Peigne CM, Leger A, Crooks JE, Konczak F, Gesnel MC, et al. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vgamma9Vdelta2 T cells. Immunity2014 Apr 17;40(4):490-500.

9. Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells. Nat Immunol2013 Sep;14(9):908-16.

10. Ravens S, Schultze-Florey C, Raha S, Sandrock I, Drenker M, Oberdorfer L, et al. Human [gamma][delta] T cells are quickly reconstituted after stem-cell

transplantation and show adaptive clonal expansion in response to viral infection. Nat Immunol2017;18(4):393-401.

11. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med2003 Jan 20;197(2):163-8.

12. Pauza CD, Cairo C. Evolution and function of the TCR Vgamma9 chain repertoire: It's good to be public. Cell Immunol2015 Jul;296(1):22-30.

13. Gomes AQ, Correia DV, Grosso AR, Lanca T, Ferreira C, Lacerda JF, et al. Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells. Haematologica2010 Aug;95(8):1397-404.

14. Girlanda S, Fortis C, Belloni D, Ferrero E, Ticozzi P, Sciorati C, et al. MICA expressed by multiple myeloma and monoclonal gammopathy of undetermined significance plasma cells Costimulates pamidronate-activated gammadelta lymphocytes. Cancer Res2005 Aug 15;65(16):7502-8.

15. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D ligand ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells through both TCRgammadelta and NKG2D. Blood2009 Jul 09;114(2):310-7.

16. Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier JF, Scotet E, et al. V gamma 9V delta 2 T cell response to colon carcinoma cells. J Immunol2005 Oct 15;175(8):5481-8.

17. Toutirais O, Cabillic F, Le Friec G, Salot S, Loyer P, Le Gallo M, et al. DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vγ9Vδ2 T cells. European Journal of Immunology2009;39(5):1361-8.

18. Correia DV, Lopes A, Silva-Santos B. Tumor cell recognition by gammadelta T lymphocytes: T-cell receptor vs. NK-cell receptors. Oncoimmunology2013 Jan 01;2(1):e22892.

19. Hannani D, Ma Y, Yamazaki T, Dechanet-Merville J, Kroemer G, Zitvogel L. Harnessing gammadelta T cells in anticancer immunotherapy. Trends Immunol2012 May;33(5):199-206.

20. Kunzmann V, Bauer E, Wilhelm M. Gamma/delta T-cell stimulation by pamidronate. N Engl J Med1999 Mar 04;340(9):737-8.

21. Lo Presti E, Dieli F, Meraviglia S. Tumor-Infiltrating gammadelta T Lymphocytes: Pathogenic Role, Clinical Significance, and Differential Programing in the Tumor Microenvironment. Front Immunol2014;5:607.

22. Tosolini M, Pont F, Poupot M, Vergez F, Nicolau-Travers ML, Vermijlen D, et al. Assessment of tumor-infiltrating TCRVgamma9Vdelta2 gammadelta lymphocyte abundance by deconvolution of human cancers microarrays. Oncoimmunology2017;6(3):e1284723.

23. Rodrigues dos Santos C, Fonseca I, Dias Sr, Mendes de Almeida JC. Plasma level of LDL-cholesterol at diagnosis is a predictor factor of breast tumor progression. BMC Cancer2014;14:132-.

24. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol2011;12(3):204-12.

25. Kidani Y, Bensinger SJ. Modulating Cholesterol Homeostasis to Build a Better T Cell. Cell Metabolism 2017/05/22;23(6):963-4.

26. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. Immunity2007 Aug;27(2):334-48.

27. Molnar E, Swamy M, Holzer M, Beck-Garcia K, Worch R, Thiele C, et al. Cholesterol and sphingomyelin drive ligand-independent T-cell antigen receptor nanoclustering. J Biol Chem2012 Dec 14;287(51):42664-74.

28. Yang W, Bai Y, Xiong Y, Zhang J, Chen S, Zheng X, et al. Potentiating the antitumour response of CD8+ T cells by modulating cholesterol metabolism. Nature2016;531(7596):651-5.

29. Sag D, Cekic C, Wu R, Linden J, Hedrick CC. The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. Nature Communications2015;6:6354.

30. Rodrigues dos Santos C, Domingues G, Matias Is, Matos Jo, Fonseca I, de Almeida JM, et al. LDL-cholesterol signaling induces breast cancer proliferation and invasion. Lipids in Health and Disease. [journal article]. 2014;13(1):16.

31. Rooney JP, Ryde IT, Sanders LH, Howlett EH, Colton MD, Germ KE, et al. PCR based determination of mitochondrial DNA copy number in multiple species. Methods Mol Biol2015;1241:23-38.

32. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. Cell Metab2016 Jan 12;23(1):27-47.

33. Beloribi-Djefaflia S, Vasseur S, Guillaumond F. Lipid metabolic reprogramming in cancer cells. Oncogenesis2016;5:e189.

34. Havas KM, Milchevskaya V, Radic K, Alladin A, Kafkia E, Garcia M, et al. Metabolic shifts in residual breast cancer drive tumor recurrence. The Journal of Clinical Investigation2017;127(6).

35. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer2007 Oct;7(10):763-77.

36. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. Nat Med2011 Oct 30;17(11):1498-503.

37. Uehara H, Takahashi T, Oha M, Ogawa H, Izumi K. Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression. Int J Cancer2014 Dec 01;135(11):2558-68.

38. Condamine T, Dominguez GA, Youn J-I, Kossenkov AV, Mony S, Alicea-Torres K, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. Science Immunology2016;1(2):aaf8943-aaf.

39. Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S, Howe MK, et al. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. Science2013 Nov 29;342(6162):1094-8.

40. Lingwood D, Simons K. Lipid Rafts As a Membrane-Organizing Principle. Science2010;327(5961):46-50.

41. Sag D, Cekic C, Wu R, Linden J, Hedrick CC. The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. Nat Commun2015 Feb 27;6:6354.

42. Zhang K, Kaufman RJ. Unfolding the toxicity of cholesterol. Nat Cell Biol2003;5(9):769-70.

43. Pan Y, Tian T, Park CO, Lofftus SY, Mei S, Liu X, et al. Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. Nature2017 Mar 09;543(7644):252-6.

44. Bengsch B, Johnson AL, Kurachi M, Odorizzi PM, Pauken KE, Attanasio J, et al. Bioenergetic Insufficiencies Due to Metabolic Alterations Regulated by the Inhibitory Receptor PD-1 Are an Early Driver of CD8+ T Cell Exhaustion. Immunity2016;45(2):358-73.

45. Scharping NE, Menk AV, Moreci RS, Whetstone RD, Dadey RE, Watkins SC, et al. The Tumor Microenvironment Represses T Cell Mitochondrial Biogenesis to Drive Intratumoral T Cell Metabolic Insufficiency and Dysfunction. Immunity2016 Sep 20;45(3):701-3.

46. Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. Immunity2009 Aug 21;31(2):184-96.

47. Ramana CV, Gil MP, Schreiber RD, Stark GR. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. Trends Immunol2002 Feb;23(2):96-101.

48. Arkko S, Zlatev HP, Monkkonen H, Raikkonen J, Benzaid I, Clezardin P, et al. Upregulation of the mevalonate pathway by cholesterol depletion abolishes tolerance to N-bisphosphonate induced Vgamma9Vdelta2 T cell cytotoxicity in PC-3 prostate cancer cells. Cancer Lett2015 Feb 01;357(1):279-85.

49. Tie G, Yan J, Khair L, Messina JA, Deng A, Kang J, et al. Hypercholesterolemia Increases Colorectal Cancer Incidence by Reducing Production of NKT and gammadelta T Cells from Hematopoietic Stem Cells. Cancer Res2017 May 01;77(9):2351-62.

50. McCaw L, Shi Y, Wang G, Li Y-J, Spaner DE. Low Density Lipoproteins Amplify Cytokine-signaling in Chronic Lymphocytic Leukemia Cells. EBioMedicine2017;15:24-35.

3. Conclusion and Future perspectives

This PhD thesis describes for the first time, to our knowledge, the role of LDL-cholesterol as an important inhibitor of γδ T cell functions. Furthermore, it paves the way for further studies on the impact of systemic cholesterol in the success of cellular immunotherapies.

The tumor microenvironment is composed of network of immune and endothelial and stromal cells embedded in the extracellular matrix under specific physiochemical conditions (e.g., lipids, acidic pH and hypoxia)[150,151]. A majority of tumors are normally treated by agents causing DNA damage as the chemotherapies and radiotherapy. However, the therapeutic index of these treatments is limit because of the intrinsic or acquired resistance of tumors and toxicity of treatment on healthy tissues, which limits the using dose. In contrast, immunotherapies have been used as an alternative therapeutic strategy and have already shown promising results. The concept of immune surveillance ensures that a physiological function of the immune system is to recognize and destroy transformed cell clones before they become tumors, and to destroy formed tumors[152]. This concept was proposed in 1950 by McFarlane Burnet and served as a basis for the development of alternative treatments, such as antineoplastic immunotherapy, with very promising results for treatments of tumors such as kidney, lung, and melanomas that did not respond well to chemotherapy[153, 154,155 156] Similar to Correia and colleagues, here we show that γδ T cells from healthy donors when activated with IL-2 and phosphoantigen-PP -displayed efficient anti-tumor activity[72,157]. Surprisingly, we observed that in the presence of LDL-cholesterol, the activation and anti-tumor functions of γδ T cells were significantly inhibited, as illustrated by decreases in the following parameters: CD69 expression, intracellular IFN-γ production, STAT-1 (transcription factor, that play a predominant role in most of the major biological activities of IFN-γ), NKG2D, DNAM-1 and CD56 (identifies highly cytotoxic cells) levels; and in vitro and vivo killing of MDA231 breast cancer cells. Our data further demonstrated that NKG2D and DNAM-1 are the main cytotoxicity receptors involved in breast cancer cells killing by human Vδ2^{+} γδ T cells (Figure 7).

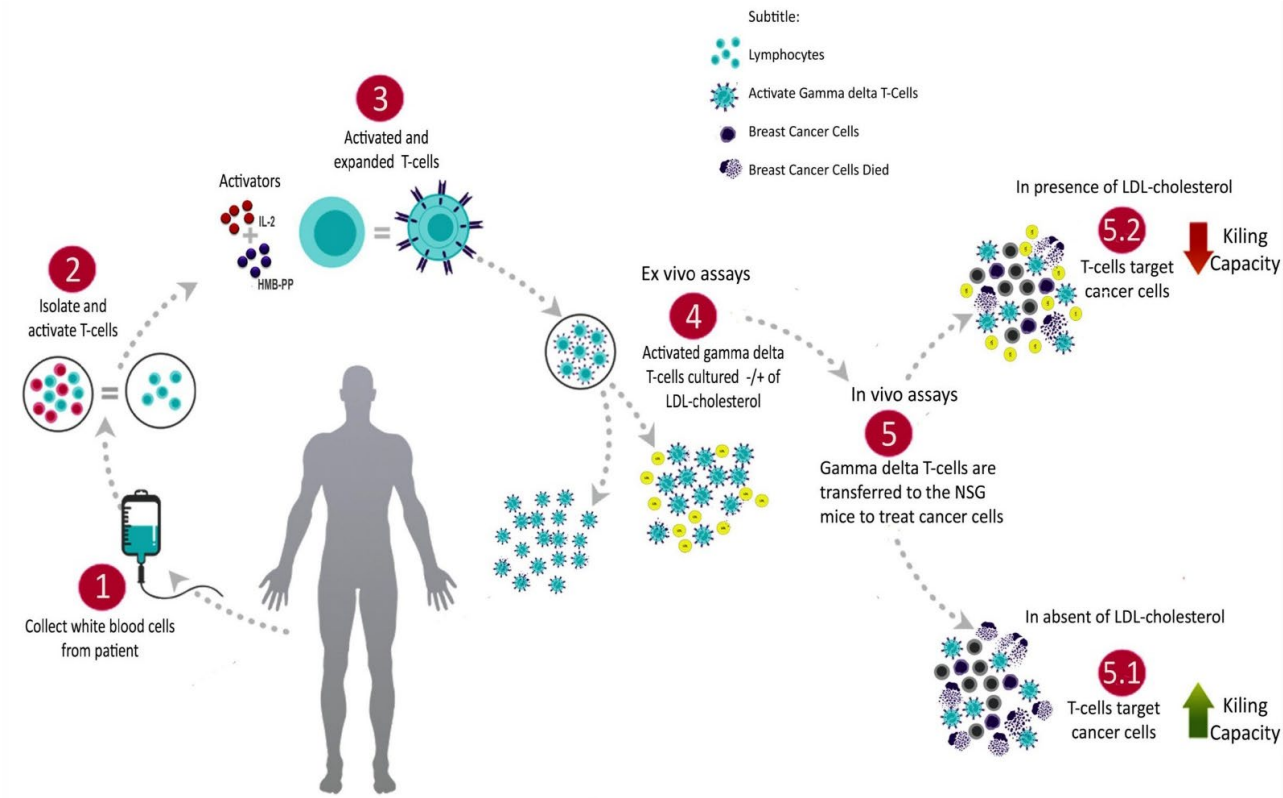


Figure 7: Schematic of the approach and conclusions of this thesis: LDL-cholesterol limits the anti-tumor activity of human γδ T cells.

Hyperlipidemia, and in particular hypercholesterolemia (abnormally elevated systemic levels of lipoproteins namely LDL-cholesterol), has shown an increased incidence and prevalence up to the present day [158]. This condition has been shown to contribute towards increasing the incidence of several chronic diseases. For instance, several studies demonstrated that the accumulation of cholesterol in macrophage foam cells results from the uptake of modified apoB lipoproteins leading to a chronic inflammatory disease termed atherogenesis [159,160,161,162,105]. In this context, it has also been recently recognized that the recruitment of monocytes and other leukocytes into the artery may be critical for disease regression and inflammation resolution[163]. The resultant dysregulation of lipid metabolism alters the macrophage phenotype and compromises critical immune responses. In our study, we used animals with elevated blood LDL cholesterol to analyze the

functionality of their Vγ9Vδ2 T cells. We also addressed the impact of LDL-cholesterol in the widely used syngeneic E0771 model of breast cancer, where endogenous γδ T cells can be exposed to high-fat diet *in vivo*. The results clearly show an impact on γδ T cell functions, particularly the co-production of the key anti-tumor cytokines, IFN-γ and TNF-α. In addition, it is known obesity is also characterized by overproduction of inflammatory cytokines derived from adipocytes such as TNF alpha and interleukin-1 which can lead to macrophage activation via GM-CSF[148].

On the other hand, some aspects of cancer behaviour, such as tissue invasion and metastasis formation, may be affected by similar mechanisms. In fact, studies, have suggested that metabolic alterations play a pivotal role during tumor development [151,150]. In detail, high cholesterol levels have been associated with breast cancer development and it is know that tumor cells have increased cholesterol uptake, believed to be due to their increased proliferative rate and the demand for increased membrane synthesis [164, 147].

Our observations, showing the negative effects of LDL cholesterol on anti-tumor functions of gamma delta were reversed using cholesterol inhibitors including nystatin and anti-LDL-R. It has been demonstrated that, high intracellular cholesterol levels decrease the activity of HMG-coa reductase and LDL receptor expression, by negative feedback. On the other hand, with lower levels of cholesterol in circulation, de novo synthesis is activated and more LDL receptors are exposed on surface of cell membranes. Importantly, these are fully consistent with our data showing that Vγ9Vδ2 T cells require activation to express LDL-R, upon which LDL uptake interferes with the activation state and inhibits anti-tumor functions, thus potentially compromising the success of adoptive Vγ9Vδ2 T cell therapy.

In contrast, one study suggested that inhibition of cholesterol esterification with ACAT inhibitor-avasimibe increased the anti-tumor capacity of CD8 T cells. These studies further suggested that the combined therapy of avasimibe plus an anti-PD-1 antibody may be effective at controlling tumor development[119]. Moreover, macrophage cholesterol efflux is a central step in reverse cholesterol transport,

which acts to maintain cholesterol homeostasis and to decrease atherosclerosis [163]. Furthermore, other studies showed higher expression of LDL-R in tumor cells when compared to normal cells[165],[166],[167]. Overall, these studies confirm the complex role of LDL-cholesterol in the tumor microenvironment. Moreover, this probably constitutes an important limitation to current T cell–mediated immunotherapy strategies.

In addition to this, antineoplastic immunotherapy focuses on a set of strategies, which aim at stimulating or improving the immune response against tumors, but this response may fail due to the several obstacles in the tumor microenvironment. In more detail, several types of immunotherapy are used to treat cancer, including T cell therapy, oncolytic viruses, vaccine therapy, tumor-agnostic therapies and monoclonal antibodies. T-cell therapy (including the use of CAR-T cells), usually requires T cells isolated from the patient, which are genetically modified and have been used in the treatment of patients with acute lymphoblastic leukemia (ALL). For instance[168,169] Oncolytic virus therapy, involves the use of oncolytic viruses such as the laimparepvec (Imlygic) or T-VEC talimogene, have been used to kill cancer cells such as melanoma and for the treatment of brain tumors. Preclinical studies have shown promising results for oncolytic viruses engineered with tumor-associated antigens (TAAs) and those combined with checkpoint inhibitors (e.g., CAPRA, CAPTIVE, and Masterkey-265) or other immunemodulators or cell therapies[170,171]. Vaccine-based therapies are used to prevent cancer promotion by human papilloma virus infection [172,173,174]. Non-specific immunotherapies, using interleukin 2 provided early clinical evidence of the potential of immunotherapy in a small subset of melanoma patients. Immunotherapy with checkpoint inhibitors has emerged as a novel approach to treat several solid tumors. Hodi and colleagues demonstrated that treatment with ipilimumab, an antibody that targets the T cell checkpoint protein CTLA-4, improved overall survival of patients with metastatic melanoma[175]. Recently the Nobel Prize in Medicine was awarded to James P. Allison and Tasuku Honjo, for their research that led to the use of monoclonal antibody therapy and tumor-agnostic therapies. Thus consecrating work of more than 20 years research and development allows us today to have access to a class of drugs to treat cancer with possibly fewer side effects than other types of

treatment. The studies demonstrated that one of the mechanisms of interaction between the immune system and cancer involved the CTLA-4 and PD-1/PDL1 receptors, which are critical pathways for the immune system's ability to control the growth of cancer[153].

Still, the limited success of immunotherapy in some types of tumor is thought to be multi-factorial, influenced by an immunosuppressive tumor microenvironment and lack of tumor-associated neo-antigens to stimulate an immune response in most patients. So, many immunomodulatory injected intratumorraly showed at least benefit but unlikely to control large mass For example, MHC class I expression in advanced melanoma predict resistance to anti-CTLA-4 but not to anti-PD1[176]; Both CTLA-4 and PD-1 contribute to the attenuation of CD8-mediated costimulation[177]; Gene expression profiling shows distinct immune cell populations in response to PD-1 combined CTLA-4 plus PD-1 blockade[178], challenges of high dose interleukin 2: unfavorable therapeutic index with only about 10% durable benefits; intensive and expensive care required for management of multi-organ toxicities, especially hypotension[179,180].

Also, a major obstacle of biological therapies, due to the vast number of genetic and epigenetic changes associated with carcinogenesis, as shown in tumors expressing neoantigens, including malignant melanoma lymphoma, colon cancer, osteosarcoma, breast cancer, prostate cancer, Burkitt lymphoma, neuroblastoma, lung carcinomas, leads to their ability to evade or overcome host defense mechanisms[155]. However, it is clear that T-cell therapy represents an emerging and promising modality for the treatment of cancer. Currently, increasing importance of gamma delta cells and their products in the neoplastic tissue, for the following reasons: Vδ2γ9 T cells can be used as promising targets of novel anti-tumor therapies due to their function plasticity: anti-tumor activity without MHC or CD1 dependent presentations[2]; cell receptor mediated recognition of aminobisphosphate[181]; express natural killer receptor and produces cytokines, such as IFNg and TNF-alpha. Clinical trial showed that patients treated with adoptive transfer of γδ T cells in combination with IL-2, had efficiently delay growth of malignantly transformed cells[182]. However, biochemical pathways that

link antigen recognition with T cell activation are poorly understood. Notably, in vivo assays, gamma delta T cells accumulated cholesterol, reducing their capacity to be activated, which contributes to failure to resolve inflammation and control tumor progression. These data suggest higher LDL-cholesterol has tumor suppressive functions in breast cancer, by enhancing the ability of tumors to escape the action of γδ T cells via downregulation of two principal cytotoxic receptors, DNAM-1 and NKG2D.

Our study opens the way for new approaches to cancer immunotherapy in hypercholesterolemic patients. For future studies it will be relevant to use LDL receptor knockout mice (in syngeneic models); or use LDL inhibitors, nystatin and anti-LDL-R mAb, to validate our *in vitro* results (Figure 8).

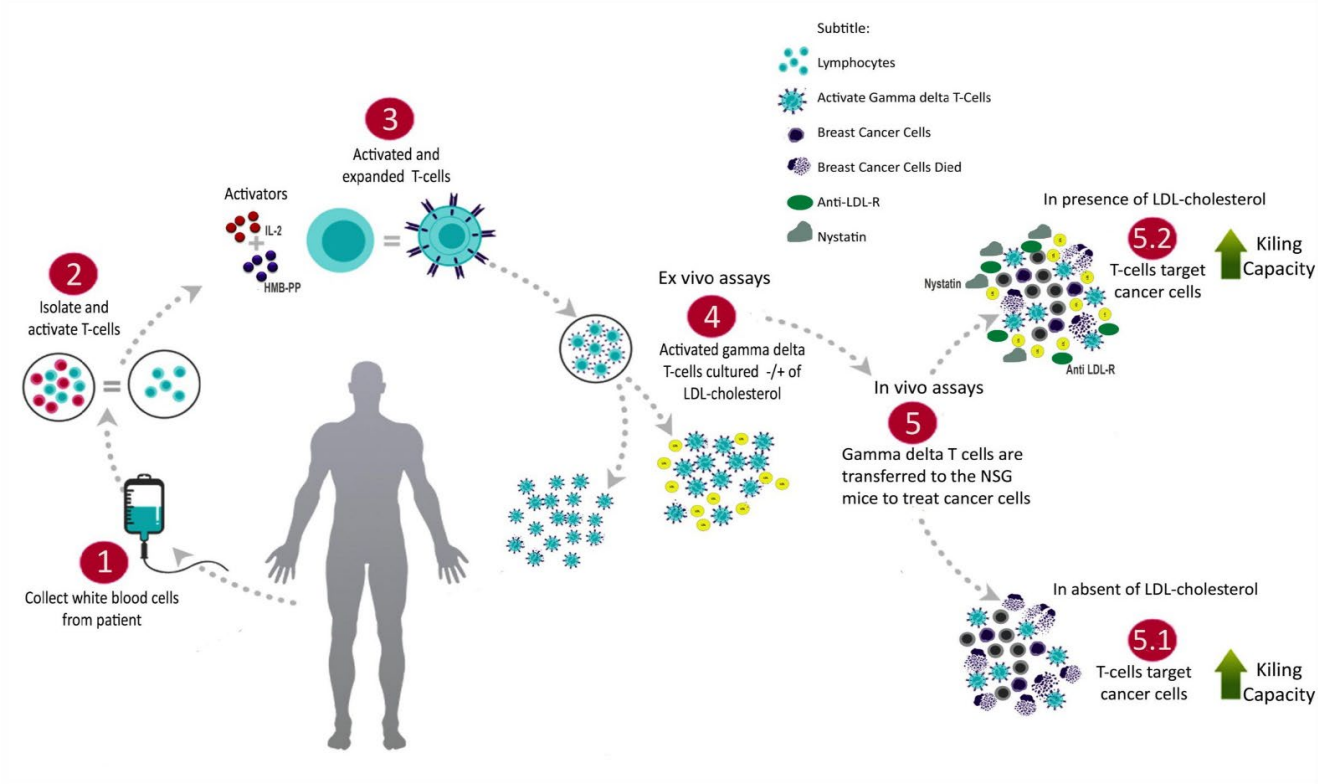


Figure 8: Future plans: Intending to validate *in vivo* the results obtained *in vitro* in terms of the restoration, via anti-LDL-R mAb or nystatin treatment, of cytotoxic receptor expression and γδ T cell anti-tumor activity in breast cancer in the presence LDL-cholesterol.

4.References

1. Hayday AC. γδ Cells: A Right Time and a Right Place for a Conserved Third Way of Protection. *Annu Rev Immunol*. 2002;18(1):975-1026. doi:10.1146/annurev.immunol.18.1.975

2. Hayday AC. γδ Cells: A Right Time and a Right Place for a Conserved Third Way of Protection. *Annu Rev Immunol*. 2000;18(1):975-1026. doi:10.1146/annurev.immunol.18.1.975

3. Nedellec S, Bonneville M, Scotet E. Human Vgamma9Vdelta2 T cells: from signals to functions. *Semin Immunol*. 2010;22(4):199-206. doi:10.1016/j.smim.2010.04.004

4. Hannani D, Ma Y, Yamazaki T, Dechanet-Merville J, Kroemer G, Zitvogel L. Harnessing gammadelta T cells in anticancer immunotherapy. *Trends Immunol*. 2012;33(5):199-206. doi:S1471-4906(12)00007-5 [pii]\r10.1016/j.it.2012.01.006

5. Vavassori S, Kumar A, Wan GS, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human γδ T cells. *Nat Immunol*. 2013;14(9):908-916. doi:10.1038/ni.2665

6. Jemal. Global cancer statistics. *CA Cancer J Clin*. 1999;49(2):1,33-64. doi:10.3322/caac.20107.Available

7. Singh B, Cook KR, Martin C, et al. Evaluation of a CXCR4 antagonist in a xenograft mouse model of inflammatory breast cancer. *Clin Exp Metastasis*. 2010;27(4):233-240. doi:10.1007/s10585-010-9321-4

8. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013

9. Likis FE. Revisiting the Hallmarks of Midwifery. *J Midwifery Women's Heal*. 2010;55(1):1. doi:10.1016/j.jmwh.2009.11.004

10. Maxwell KN, Wubbenhorst B, Wenz BM, et al. BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers. *Nat Commun*. 2017;8(1). doi:10.1038/s41467-017-00388-9

11. Otto AM. Warburg effect(s)—a biographical sketch of Otto Warburg and his impacts on tumor metabolism. *Cancer Metab*. 2016;4(1):5. doi:10.1186/s40170-016-0145-9

12. Shacter E, Weitzman SA. Chronic Inflammation and Cancer. *Oncology*. 2002;16(2):1-16.

13. Selby MJ, Engelhardt JJ, Quigley M, et al. Anti-CTLA-4 Antibodies of IgG2a Isotype Enhance Antitumor Activity through Reduction of Intratumoral Regulatory T Cells. *Cancer Immunol Res*. 2013;1(1):32-42. doi:10.1158/2326-6066.CIR-13-0013

14. Beloribi-Djefaflia S, Vasseur S, Guillaumond F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis*. 2016;5(1):e189. doi:10.1038/oncsis.2015.49

15. Rodrigues Dos Santos C, Domingues G, Matias I, et al. LDL-cholesterol signaling induces breast cancer proliferation and invasion. *Lipids Health Dis*. 2014;13(1). doi:10.1186/1476-511X-13-16

16. Cairns R, Harris I, Mak T. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11(2):85-95. doi:10.1038/nrc2981

17. Goossens P, Juan Rodriguez-Vita, Etzerodt A, et al. Membrane Cholesterol Efflux Drives Tumor-Associated Macrophage Reprogramming and Tumor Progression. *Cell Metab*. 2019;0(1932-7420).

18. Graffi A. CHLOROLEUKEMIA OF MICE. *Ann N Y Acad Sci*. 1957;68(2):540-558. doi:10.1111/j.1749-6632.1957.tb56107.x

19. Svane IM, Engel AM, Nielsen M, Werdelin O. Interferon-gamma-induced MHC class I expression and defects in Jak/Stat signalling in methylcholanthrene-induced sarcomas. *Scand J Immunol*. 1997;46(4):379-387. doi:10.1046/j.1365-3083.1997.d01-141.x

20. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon □-dependent tumor surveillance system in immunocompetent mice. *Immunology*. 1998;95(June):7556-7561. doi:10.1073/pnas.95.13.7556

21. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science*. 1974;183(4124):534-536. doi:10.1126/science.134.3478.527

22. Shankaran V, Ikeda H, Bruce AT, et al. IFNγ, and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410(6832):1107-1111. doi:10.1038/35074122

23. Smyth MJ. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol*. 2008;20(4):631. doi:10.1093/intimm/dxn028

24. Girardi M, Glusac E, Filler RB, et al. The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T cells to different stages of chemically induced skin cancer. *J Exp Med*. 2003;198(5):747-755. doi:10.1084/jem.20021282

25. Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILS) in breast cancer: Recommendations by an International TILS Working Group 2014. *Ann Oncol*. 2015;26(2):259-271. doi:10.1093/annonc/mdu450

26. Pachmann K, Camara O, Kavallaris A, et al. Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. *J Clin Oncol*. 2008;26(8):1208-1215. doi:10.1200/JCO.2007.13.6523

27. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. 2015;21(8):938-945. doi:10.1038/nm.3909

28. Kitamura T, Qian BZ, Pollard JW. Immune cell promotion of metastasis. *Nat Rev Immunol*. 2015;15(2):73-86. doi:10.1038/nri3789

29. Shang B, Liu Y, Jiang S, Liu Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep*. 2015;5:15179. doi:10.1038/srep15179

30. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence,

and survival in epithelial ovarian cancer. *N Engl J Med*. 2003;348(3):203-213. doi:10.1056/NEJMoa020177

31. Pagès F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med*. 2005;353(25):2654-2666. doi:10.1056/NEJMoa051424

32. Vantourout P, Hayday A. Six-of-the-best: Unique contributions of γδ T cells to immunology. *Nat Rev Immunol*. 2013;13(2):88-100. doi:10.1038/nri3384

33. Van Acker HH, Anguille S, Van Tendeloo VF, Lion E. Empowering gamma delta T cells with antitumor immunity by dendritic cell-based immunotherapy. *Oncoimmunology*. 2015;4(8). doi:10.1080/2162402X.2015.1021538

34. Allison TJ, Winter CC, Fournie JJ, Bonneville M, Garboczi DN. Structure of a human gammadelta T-cell antigen receptor. *Nature*. 2001;411(6839):820-824. doi:10.1038/35081115

35. Human E, Hamann BD, Baars PA, et al. Phenotypic and Functional Separation of Memory and. *J Exp Med*. 1997;186(9):1407–1418.

36. Ménoret S, Guillonneau C, Bezié S, Caron L, Anegon I, Li X-L. Phenotypic and functional characterization of CD8(+) T regulatory cells. *Methods Mol Biol*. 2011;677:63-83. doi:10.1007/978-1-60761-869-0_5

37. Mallard E, Vernel-Pauillac F, Velu T, et al. IL-2 production by virus- and tumor-specific human CD8 T cells is determined by their fine specificity. *J Immunol*. 2004;172(6):3963-3970.

38. Hamann D, Roos MT, van Lier RA, et al. Faces and phases of human CD8 T-cell development\rPhenotypic and functional separation of memory and effector human CD8+ T cells. *Immunol Today*. 1999;20(4):177-80.

39. Sathaliyawala T, Kubota M, Yudanin N, et al. Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets. *Immunity*. 2013;38(1):187-197. doi:10.1016/j.immuni.2012.09.020

40. Silva-Santos B, Serre K, Norell H. γδ T cells in cancer. *Nat Rev Immunol*. 2015;15(11):683-691. doi:10.1038/nri3904

41. Tanaka Y, Morita CT, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature*. 1995;375(6527):155-158. doi:10.1038/375155a0 [doi]

42. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10(9):942-949. doi:10.1038/nm1093

43. Willcox BE, Willcox CR. γδ TCR ligands: the quest to solve a 500-million-year-old mystery. *Nat Immunol*. 2019. doi:10.1038/s41590-018-0304-y

44. Marlin R, Pappalardo A, Kaminski H, et al. Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2. *Proc Natl Acad Sci*. 2017. doi:10.1073/pnas.1621052114

45. Bruder J, Siewert K, Obermeier B, et al. Target specificity of an autoreactive pathogenic human γδ-T cell receptor in myositis. *J Biol Chem*. 2012. doi:10.1074/jbc.M112.356709

46. Silva-Santos B, Schamel WWA, Fisch P, Eberl M. γδ T-cell conference 2012: Close encounters for the fifth time. In: *European Journal of Immunology*. ; 2012. doi:10.1002/eji.201270101

47. Roy S, Ly D, Castro CD, et al. Molecular Analysis of Lipid-Reactive Vδ1 γδ T Cells Identified by CD1c Tetramers. *J Immunol*. 2016. doi:10.4049/jimmunol.1502202

48. Harly C, Guillaume Y, Nedellec S, et al. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset. *Blood*. 2012;120(11):2269-2279. doi:10.1182/blood-2012-05-430470

49. Eberl M, Altincicek B, Kollas AK, et al. Accumulation of a potent γδ T-cell stimulator after deletion of the lytB gene in Escherichia coli. *Immunology*. 2002;106(2):200-211. doi:10.1046/j.1365-2567.2002.01414.x

50. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: Discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev*. 2007;215(1):59-76. doi:10.1111/j.1600-065X.2006.00479.x

51. Morita CT, Lee HK, Wang H, Li H, Mariuzza RA, Tanaka Y. Structural Features of Nonpeptide Prenyl Pyrophosphates That Determine Their Antigenicity for Human T Cells. *J Immunol*. 2001;167(1):36-41. doi:10.4049/jimmunol.167.1.36

52. Kunzmann V, Bauer E, Feurle J, et al. Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood*. 2000;96(2):384-392. doi:10.1182/blood.v98.1.210

53. Pang DJ, Neves JF, Sumaria N, Pennington DJ. Understanding the complexity of γδ T-cell subsets in mouse and human. *Immunology*. 2012;136(3):283-290. doi:10.1111/j.1365-2567.2012.03582.x

54. Vermijlen D, Ellis P, Langford C, et al. Distinct Cytokine-Driven Responses of Activated Blood T Cells: Insights into Unconventional T Cell Pleiotropy. *J Immunol*. 2007;178(7):4304-4314. doi:10.4049/jimmunol.178.7.4304

55. Shen Y, Zhou D, Qiu L, et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. *Science*. 2002;295(5563):2255-2258. doi:10.1126/science.1068819

56. Chen ZW. Multifunctional immune responses of HMBPP-specific Vγ2Vδ2 T cells in M. tuberculosis and other infections. *Cell Mol Immunol*. 2013;10(1):58-64. doi:10.1038/cmi.2012.46

57. Harly C, Peigné CM, Scotet E. Molecules and mechanisms implicated in the peculiar antigenic activation process of human Vγ9Vδ2 T cells. *Front Immunol*. 2015;6(JAN). doi:10.3389/fimmu.2014.00657

58. Sandstrom A, Peigné CM, Léger A, et al. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2T Cells. *Immunity*. 2014;40(4):490-500.

doi:10.1016/j.immuni.2014.03.003

59. Wang H, Sarikonda G, Puan K-J, et al. Indirect Stimulation of Human Vγ2Vδ2 T Cells through Alterations in Isoprenoid Metabolism. *J Immunol*. 2011;187(10):5099-5113. doi:10.4049/jimmunol.1002697

60. Allison JP, Havran WL. The immunobiology of T cells with invariant γδ antigen receptors. *Annu Rev Immunol*. 1991;9(1):679-705. doi:10.1146/annurev.iy.09.040191.003335

61. Vilmer E, Triebel F, David V, et al. Prominent expansion of circulating lymphocytes bearing ? T-cell receptors, with preferential expression of variable ? genes after allogeneic bone marrow transplantation. *Blood*. 1988;72(3).

62. Ngoenkam J, Schamel WW, Pongcharoen S. Selected signalling proteins recruited to the T-cell receptor–CD3 complex. *Immunology*. 2018;153(1):42-50. doi:10.1111/imm.12809

63. Anderson P, Caligiuri M, Ritz J, Schlossman SF. CD3-negative natural killer cells express ε TCR as part of a novel molecular complex. *Nature*. 1989;341(6238).

64. Gu Y, Chae HD, Siefring JE, Jasti AC, Hildeman DA, Williams DA. RhoH GTPase recruits and activates Zap70 required for T cell receptor signaling and thymocyte development. *Nat Immunol*. 2006;7(11):1182-1190. doi:10.1038/ni1396

65. Rao A, Luo C, Hogan PG. TRANSCRIPTION FACTORS OF THE NFAT FAMILY:Regulation and Function. *Annu Rev Immunol*. 2002. doi:10.1146/annurev.immunol.15.1.707

66. Wu Y, Borde M, Heissmeyer V, et al. FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell*. 2006. doi:10.1016/j.cell.2006.05.042

67. Acuto O, Michel F. CD28-mediated co-stimulation: A quantitative support for TCR signalling. *Nat Rev Immunol*. 2003;3(12):939-951. doi:10.1038/nri1248

68. Prajapati K, Perez C, Rojas LBP, Burke B, Guevara-Patino JA. Functions of NKG2D in CD8+ T cells: an opportunity for immunotherapy. *Cell Mol Immunol*. 2018. doi:10.1038/cmi.2017.161

69. Zhang J, Basher F, Wu JD. NKG2D ligands in tumor immunity: Two sides of a coin. *Front Immunol*. 2015;6(MAR). doi:10.3389/fimmu.2015.00097

70. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol*. 2003;3(10):781-790. doi:10.1038/nri1199

71. González S, López-Soto A, Suarez-Alvarez B, López-Vázquez A, López-Larrea C. NKG2D ligands: key targets of the immune response. *Trends Immunol*. 2008;29(8):397-403. doi:10.1016/j.it.2008.04.007

72. Correia D V., Lopes AC, Silva-Santos B. Tumor cell recognition by γδ T lymphocytes. *Oncoimmunology*. 2013;2(1):e22892. doi:10.4161/onci.22892

73. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci*. 2001;98(20):11521-11526. doi:10.1073/pnas.201238598

74. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*. 2001;413(6852):165-171. doi:10.1038/35093109

75. Shibuya A, Campbell D, Hannum C, et al. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity*. 1996;4(6):573-581. doi:10.1016/S1074-7613(00)70060-4

76. Long EO. Versatile signaling through NKG2D. *Nat Immunol*. 2002;3(12):1119-1120. doi:10.1038/ni1202-1119

77. Liu J, Qian X, Chen Z, et al. Crystal structure of cell adhesion molecule nectin-2/CD112 and its binding to immune receptor DNAM-1/CD226. *J Immunol*. 2012;188(11):5511-5520. doi:10.4049/jimmunol.1200324

78. Mistry AR, O'Callaghan CA. Regulation of ligands for the activating receptor NKG2D. *Immunology*. 2007;121(4):439-447. doi:10.1111/j.1365-

2567.2007.02652.x

79. Xiong P, Sang HW, Zhu M. Critical roles of co-activation receptor DNAX accessory molecule-1 in natural killer cell immunity. *Immunology*. 2015;146(3):369-378. doi:10.1111/imm.12516

80. Belardelli F, Ferrantini M, Proietti E, Kirkwood JM. Interferon-alpha in tumor immunity and immunotherapy. *Cytokine Growth Factor Rev*. 2002;13(2):119-134. doi:10.1016/S1359-6101(01)00022-3

81. Dieli F, Vermijlen D, Fulfaro F, et al. Targeting human gamma delta T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res*. 2007;67(15):7450-7457. doi:10.1158/0008-5472.can-07-0199

82. Meraviglia S, Eberl M, Vermijlen D, et al. In vivo manipulation of V9V2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. *Clin Exp Immunol*. 2010;161(2):290-297. doi:10.1111/j.1365-2249.2010.04167.x

83. Kobayashi H, Tanaka Y, Shimmura H, Minato N, Tanabe K. Complete remission of lung metastasis following adoptive immunotherapy using activated autologous ???? T-cells in a patient with renal cell carcinoma. *Anticancer Res*. 2010;30(2):575-579.

84. Nedellec S, Bonneville M, Scotet E. Human Vγ9Vδ2 T cells: From signals to functions. *Semin Immunol*. 2010;22(4):199-206. doi:10.1016/j.smim.2010.04.004

85. Kabelitz D, Wesch D, He W. Perspectives of γδ T Cells in Tumor Immunology: Figure 1. *Cancer Res*. 2007;67(1):5-8. doi:10.1158/0008-5472.CAN-06-3069

86. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: Current status and future perspectives. *Nat Rev Clin Oncol*. 2012. doi:10.1038/nrclinonc.2011.177

87. Ferrarini M, Ferrero E, Dagna L, Poggi A, Zocchi MR. Human γδ T cells: A nonredundant system in the immune-surveillance against cancer. *Trends Immunol*. 2002;23(1):14-18. doi:10.1016/S1471-4906(01)02110-X

88. Gajewski TF. The Expanding Universe of Regulatory T Cell Subsets in Cancer. *Immunity*. 2007;27(2):185-187. doi:10.1016/j.immuni.2007.08.001

89. Nahrendorf M, Swirski FK. Neutrophil-macrophage communication in inflammation and atherosclerosis. *Science (80-)*. 2015;349(6245):237-238. doi:10.1126/science.aac7801

90. Kritikou E. Metabolism: Warburg effect revisited. *Nat Rev Cancer*. 2008;8(4):247. doi:10.1038/nrc2364

91. Chistiakov DA, Bobryshev Y V., Orekhov AN. Macrophage-mediated cholesterol handling in atherosclerosis. *J Cell Mol Med*. 2016;20(1):17-28. doi:10.1111/jcmm.12689

92. Linsel-Nitschke P, Jansen H, Aherrarhou Z, et al. Macrophage cholesterol efflux correlates with lipoprotein subclass distribution and risk of obstructive coronary artery disease in patients undergoing coronary angiography. *Lipids Health Dis*. 2009;8. doi:10.1186/1476-511X-8-14

93. OREN R, FARNHAM AE, SAITO K, MILOFSKY E, KARNOVSKY ML. Metabolic patterns in three types of phagocytizing cells. *J Cell Biol*. 1963;17:487-501. doi:10.1083/jcb.17.3.487

94. Tannahill GM, Curtis AM, Adamik J, et al. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*. 2013;496(7444):238-242. doi:10.1038/nature11986

95. O'Sullivan D, Pearce EL. Targeting T cell metabolism for therapy. *Trends Immunol*. 2015;36(2):71-80. doi:10.1016/j.it.2014.12.004

96. Berod L, Friedrich C, Nandan A, et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nat Med*. 2014;20(11). doi:10.1038/nm.3704

97. Pollizzi KN, Powell JD. Integrating canonical and metabolic signalling programmes in the regulation of T cell responses. *Nat Rev Immunol*. 2014;14(7):435-446. doi:10.1038/nri3701

98. Jacobs SR, Herman CE, MacIver NJ, et al. Glucose Uptake Is Limiting in T Cell

Activation and Requires CD28-Mediated Akt-Dependent and Independent Pathways. *J Immunol*. 2008;180(7):4476-4486. doi:10.4049/jimmunol.180.7.4476

99. Krawczyk CM, Holowka T, Sun J, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood*. 2010;115(23):4742-4749. doi:10.1182/blood-2009-10-249540

100. Minton K. T cell responses: Quantity and quality control by mTOR. *Nat Rev Immunol*. 2009;9(8):534. doi:10.1038/nri2609

101. Delgoffe GM, Kole TP, Zheng Y, et al. The mTOR Kinase Differentially Regulates Effector and Regulatory T Cell Lineage Commitment. *Immunity*. 2009;30(6):832-844. doi:10.1016/j.immuni.2009.04.014

102. Araki K, Turner AP, Shaffer VO, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature*. 2009;460(7251):108-112. doi:10.1038/nature08155

103. Goedeke L, Fernández-Hernando C. Regulation of cholesterol homeostasis. *Cell Mol Life Sci*. 2011;69(6):915-930. doi:10.1007/s00018-011-0857-5

104. Cyster JG, Dang E V, Reboldi A, Yi T. 25-Hydroxycholesterols in innate and adaptive immunity. *Nat Rev Immunol*. 2014;14(11):731-743. doi:10.1038/nri3755

105. Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. *Nature*. 2005;438(7068):612-621. doi:10.1038/nature04399

106. Houten SM, Watanabe M, Auwerx J. Endocrine functions of bile acids. *EMBO J*. 2006;25(7):1419-1425. doi:10.1038/sj.emboj.7601049

107. Kurzchalia T V., Ward S. Why do worms need cholesterol? *Nat Cell Biol*. 2003;5(8):684-688. doi:10.1038/ncb0803-684

108. Rosenman RH. Psychological influences on the variability of plasma cholesterol. *Homeostasis*. 1993;34:129-136.

109. McGrowder D, Riley C, Morrison EYSA, Gordon L. The role of high-density lipoproteins in reducing the risk of vascular diseases, neurogenerative disorders, and cancer. *Cholesterol*. 2011;2011. doi:10.1155/2011/496925

110. Bietz A, Zhu H, Xue M, Xu C. Cholesterol metabolism in T cells. *Front Immunol*. 2017;8(NOV). doi:10.3389/fimmu.2017.01664

111. Knott TJ, Pease RJ, Powell LM, et al. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature*. 1986;323(6090):734-738. doi:10.1038/323734a0

112. Resink T, Tkachuk V, Hahn A, Bochkov V. Activation of phosphoinositide catabolism and DNA synthesis in vascular smooth muscle cells by LDL and positively charged proteins: Evidence that effects of LDL are not specifically dependent on the polycationic nature of APO B. *Biochem Biophys Res Commun*. 1994;203(3):1700-1709. doi:10.1006/bbrc.1994.2382

113. Wang N, Silver DL, Thiele C, Tall AR. ATP-binding Cassette Transporter A1 (ABCA1) Functions as a Cholesterol Efflux Regulatory Protein. *J Biol Chem*. 2001;276(26):23742-23747. doi:10.1074/jbc.M102348200

114. Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. *Nat Rev Neurosci*. 2006;7(11):850-859. doi:10.1038/nrn2009

115. Van Der Wulp MYM, Verkade HJ, Groen AK. Regulation of cholesterol homeostasis. *Mol Cell Endocrinol*. 2013;368(1-2):1-16. doi:10.1016/j.mce.2012.06.007

116. Spann NJ, Glass CK. Sterols and oxysterols in immune cell function. *Nat Immunol*. 2013;14(9):893-900. doi:10.1038/ni.2681

117. Day I, Humphries S. Genetic Tests for Familial Hypercholesterolemia. *Nat Biotechnol*. 1996;14(10):1227-1228. doi:10.1038/nbt1096-1227

118. Abifadel M, Varret M, Rabès JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet*. 2003;34(2):154-156. doi:10.1038/ng1161

119. Yang W, Bai Y, Xiong Y, et al. Potentiating the antitumour response of CD8+ T cells by modulating cholesterol metabolism. *Nature*. 2016;531(7596):651-655. doi:10.1038/nature17412

120. Lin CY, Gustafsson JA. Targeting liver X receptors in cancer therapeutics. *Nat*

Rev Cancer. 2015;15(4):216-224. doi:10.1038/nrc3912

121. Castrillo A, Joseph SB, Vaidya SA, et al. Crosstalk between LXR and Toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. *Mol Cell*. 2003;12(4):805-816. doi:10.1016/S1097-2765(03)00384-8

122. Treatment C, Ctt T. Efficacy and safety of more intensive lowering of LDL cholesterol: A meta-analysis of data from 170 000 participants in 26 randomised trials. *Lancet*. 2010;376(9753):1670-1681. doi:10.1016/S0140-6736(10)61350-5

123. Samson S, Mundkur L, Kakkar V V. Immune response to lipoproteins in atherosclerosis. *Cholesterol*. 2012;2012. doi:10.1155/2012/571846

124. Mulay SR, Desai J, Kumar S V., et al. [New oxidized LDL receptors and their functions in atherogenesis]. *Nat Commun*. 2016;7(3):10274.

125. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011;145(3):341-355. doi:10.1016/j.cell.2011.04.005

126. Curtiss LK, Tobias PS. Emerging role of Toll-like receptors in atherosclerosis. *J Lipid Res*. 2009;50(Supplement):S340-S345. doi:10.1194/jlr.R800056-JLR200

127. Stienstra R, van Diepen JA, Tack CJ, et al. Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci*. 2011;108(37):15324-15329. doi:10.1073/pnas.1100255108

128. Yvan-Charvet L, Pagler T, Gautier EL, et al. ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. *Science*. 2010;328(5986):1689-1693. doi:10.1126/science.1189731

129. Condamine T, Gabrilovich DI, Dominguez GA, et al. *Lectin-Type Oxidized LDL Receptor-1 Distinguishes Population of Human Polymorphonuclear Myeloid-Derived Suppressor Cells in Cancer Patients*. Vol 1.; 2016. doi:10.1126/sciimmunol.aaf8943.Lectin-type

130. Uldrich AP, Le Nours J, Pellicci DG, et al. CD1d-lipid antigen recognition by

the γδ TCR. *Nat Immunol*. 2013;14(11):1137-1145. doi:10.1038/ni.2713

131. Tie G, Yan J, Khair L, et al. Hypercholesterolemia increases colorectal cancer incidence by reducing production of NKT and γδ T cells from hematopoietic stem cells. *Cancer Res*. 2017;77(9):2351-2362. doi:10.1158/0008-5472.CAN-16-1916

132. Ioan-Facsinay A, Kwekkeboom JC, Westhoff S, et al. Adipocyte-derived lipids modulate CD4+ T-cell function. *Eur J Immunol*. 2013;43(6):1578-1587. doi:10.1002/eji.201243096

133. de Jong AJ, Kloppenburg M, Toes REM, Ioan-Facsinay A. Fatty Acids, Lipid Mediators, and T-Cell Function. *Front Immunol*. 2014;5. doi:10.3389/fimmu.2014.00483

134. Moran EJ, Wherry MC, Miceli R, et al. Memory CD8 T Cells TCR Signal Transduction in Antigen-Specific TCR Signal Transduction in Antigen-Specific Memory CD8 T Cells. *J Immunol Ref*. 2017;1701701154:5455-5463. doi:10.4049/jimmunol.170.11.5455

135. Fahmy TM, Bieler JG, Edidin M, Schneck JP. Increased TCR Avidity after T Cell Activation A Mechanism for Sensing Low-Density Antigen. *Immunity*. 2001;14(2):135-143. doi:10.1016/S1074-7613(09)00099-5

136. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell*. 1994;76(1):17-27. doi:0092-8674(94)90169-4 [pii]

137. Xu C, Gagnon E, Call ME, et al. Regulation of T cell receptor activation by dynamic membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif. *Cell*. 2008;135(4):702-713. doi:10.1016/j.cell.2008.09.044

138. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: Energy metabolism and the T-cell response. *Nat Rev Immunol*. 2005;5(11):844-852. doi:10.1038/nri1710

139. Schamel WWA, Alarcon B, Höfer T, Minguet S. The Allostery Model of TCR Regulation. *J Immunol*. 2017;198(1):47-52. doi:10.4049/jimmunol.1601661

140. Swamy M, Beck-Garcia K, Beck-Garcia E, et al. A Cholesterol-Based Allostery Model of T Cell Receptor Phosphorylation. *Immunity*. 2016;44(5):1091-1101. doi:10.1016/j.immuni.2016.04.011

141. Mansour S, Tocheva AS, Cave-Ayland C, et al. Cholesteryl esters stabilize human CD1c conformations for recognition by self-reactive T cells. *Proc Natl Acad Sci*. 2016. doi:10.1073/pnas.1519246113

142. Peinado H, Lavotshkin S, Lyden D. The secreted factors responsible for pre-metastatic niche formation: Old sayings and new thoughts. *Semin Cancer Biol*. 2011;21(2):139-146. doi:10.1016/j.semcancer.2011.01.002

143. Nagaraj S, Collazo M, Corzo CA, et al. Regulatory myeloid suppressor cells in health and disease. *Cancer Res*. 2009;69(19). doi:10.1158/0008-5472.CAN-09-2152

144. Havas KM, Milchevskaya V, Radic K, et al. Metabolic shifts in residual breast cancer drive tumor recurrence. *J Clin Invest*. 2017;127(6):2091-2105. doi:10.1172/JCI89914

145. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer*. 2007;7(10):763-777. doi:10.1038/nrc2222

146. Nieman KM, Kenny HA, Penicka C V., et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med*. 2011;17(11):1498-1503. doi:10.1038/nm.2492

147. Uehara H, Takahashi T, Oha M, Ogawa H, Izumi K. Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression. *Int J Cancer*. 2014;135(11):2558-2568. doi:10.1002/ijc.28903

148. Nelson ER, Wardell SE, Jasper JS, et al. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. *Science (80-)*. 2013;342(6162):1094-1098. doi:10.1126/science.1241908

149. McDonnell DP, Park S, Goulet MT, et al. Obesity, cholesterol metabolism, and breast cancer pathogenesis. *Cancer Res*. 2014;74(18):4976-4982. doi:10.1158/0008-5472.CAN-14-1756

150. Martinez-Outschoorn UE, Lin Z, Ko YH, et al. Understanding the metabolic basis of drug resistance: Therapeutic induction of the Warburg effect kills cancer cells. *Cell Cycle*. 2011. doi:10.4161/cc.10.15.16584

151. Koppenol WH, Bounds PL, Dang C V. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer*. 2011. doi:10.1038/nrc3038

152. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: From immunosurveillance to tumor escape. *Nat Immunol*. 2002. doi:10.1038/ni1102-991

153. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: Toward combination strategies with curative potential. *Cell*. 2015;161(2):205-214. doi:10.1016/j.cell.2015.03.030

154. Boon T, Cerottini J, Bruggen P Van Der, Pel A Van. TUMOR ANTIGENS RECOGNIZED. *Annu Rev Immunol*. 1994.

155. Van Der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science (80-)*. 1991. doi:10.1126/science.1840703

156. Yang JC, Rosenberg SA. Adoptive T-Cell Therapy for Cancer. In: *Advances in Immunology*. ; 2016. doi:10.1016/bs.ai.2015.12.006

157. Almeida AR, Correia D V., Fernandes-Platzgummer A, et al. Delta one T cells for immunotherapy of chronic lymphocytic leukemia: Clinical-grade expansion/differentiation and preclinical proof of concept. *Clin Cancer Res*. 2016. doi:10.1158/1078-0432.CCR-16-0597

158. Nelson RH. Hyperlipidemia as a Risk Factor for Cardiovascular Disease. *Prim Care - Clin Off Pract*. 2013. doi:10.1016/j.pop.2012.11.003

159. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. *Nat Rev Immunol*. 2013. doi:10.1038/nri3520

160. Williams KJ, Tabas I. The response-to-retention hypothesis of atherogenesis reinforced. *Curr Opin Lipidol*. 1998. doi:10.1097/00041433-199810000-00012

161. Rubin EM, Krauss RM, Spangler EA, Verstuyft JG, Clift SM. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature*. 1991. doi:10.1038/353265a0

162. Kzhyshkowska J, Neyen C, Gordon S. Role of macrophage scavenger receptors in atherosclerosis. *Immunobiology*. 2012. doi:10.1016/j.imbio.2012.02.015

163. Nathan C, Ding A. Nonresolving Inflammation. *Cell*. 2010. doi:10.1016/j.cell.2010.02.029

164. C.R. S, J.M. A, S. D. Systemic low-density lipoprotein promotes breast cancer progression. *Ann Surg Oncol*. 2012;19(2):100.

165. Versluis AJ, Van Geel PJ, Oppelaar H, Van Berkel TJC, Bijsterbosch MK. Receptor-mediated uptake of low-density lipoprotein by B16 melanoma cells in vitro and in vivo in mice. *Br J Cancer*. 1996. doi:10.1038/bjc.1996.396

166. Llaverias G, Danilo C, Mercier I, et al. Role of cholesterol in the development and progression of breast cancer. *Am J Pathol*. 2011;178(1):402-412. doi:10.1016/j.ajpath.2010.11.005

167. Kuzu OF, Noory MA, Robertson GP. The role of cholesterol in cancer. *Cancer Res*. 2016. doi:10.1158/0008-5472.CAN-15-2613

168. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A*. 1993.

169. Eshhar Z, Waks T, Gross G. The Emergence of T-Bodies/CAR T Cells. *Cancer J*. 2014;20(2):123-126. doi:10.1097/PPO.0000000000000027

170. LaRocca CJ, Warner SG. Oncolytic viruses and checkpoint inhibitors: combination therapy in clinical trials. *Clin Transl Med*. 2018. doi:10.1186/s40169-018-0214-5

171. Twumasi-Boateng K, Pettigrew JL, Kwok YYE, Bell JC, Nelson BH. Oncolytic

viruses as engineering platforms for combination immunotherapy. *Nat Rev Cancer*. 2018. doi:10.1038/s41568-018-0009-4

172. Gillison ML, Chaturvedi AK, Lowy DR. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. *Cancer*. 2008. doi:10.1002/cncr.23764

173. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011. doi:10.1200/JCO.2011.36.4596

174. Naud PS, Roteli-Martins CM, De Carvalho NS, et al. Sustained efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine. *Hum Vaccin Immunother*. 2014;10(8):2147-2162. doi:10.4161/hv.29532

175. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010. doi:10.1056/NEJMoa1003466

176. Rodig SJ, Gusenleitner D, Jackson DG, et al. MHC proteins confer differential sensitivity to CTLA-4 and PD-1 blockade in untreated metastatic melanoma. *Sci Transl Med*. 2018. doi:10.1126/scitranslmed.aar3342

177. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov*. 2018. doi:10.1158/2159-8290.CD-18-0367

178. Gide TN, Quek C, Menzies AM, et al. Distinct Immune Cell Populations Define Response to Anti-PD-1 Monotherapy and Anti-PD-1/Anti-CTLA-4 Combined Therapy. *Cancer Cell*. 2019;35(2):238-255.e6. doi:10.1016/j.ccell.2019.01.003

179. Skrombolas D, Frelinger JG. Challenges and developing solutions for increasing the benefits of IL-2 treatment in tumor therapy. *Expert Rev Clin Immunol*. 2014. doi:10.1586/1744666X.2014.875856

180. Shablak A, Sikand K, Shanks JH, Thistlethwaite F, Spencer-Shaw A, Hawkins RE. High-dose interleukin-2 can produce a high rate of response and durable remissions in appropriately selected patients with metastatic renal cancer. *J*

Immunother. 2011. doi:10.1097/CJI.0b013e3181fb659f

181. Das H, Wang L, Kamath A, Bukowski JF. Vγ2Vδ2 T-cell receptor-mediated recognition of aminobisphosphonates. *Blood*. 2001. doi:10.1182/blood.V98.5.1616

182. Gomes AQ, Martins DS, Silva-Santos B. Targeting γδ T lymphocytes for cancer immunotherapy: From novel mechanistic insight to clinical application. *Cancer Res*. 2010. doi:10.1158/0008-5472.CAN-10-3236

Supplementary figures

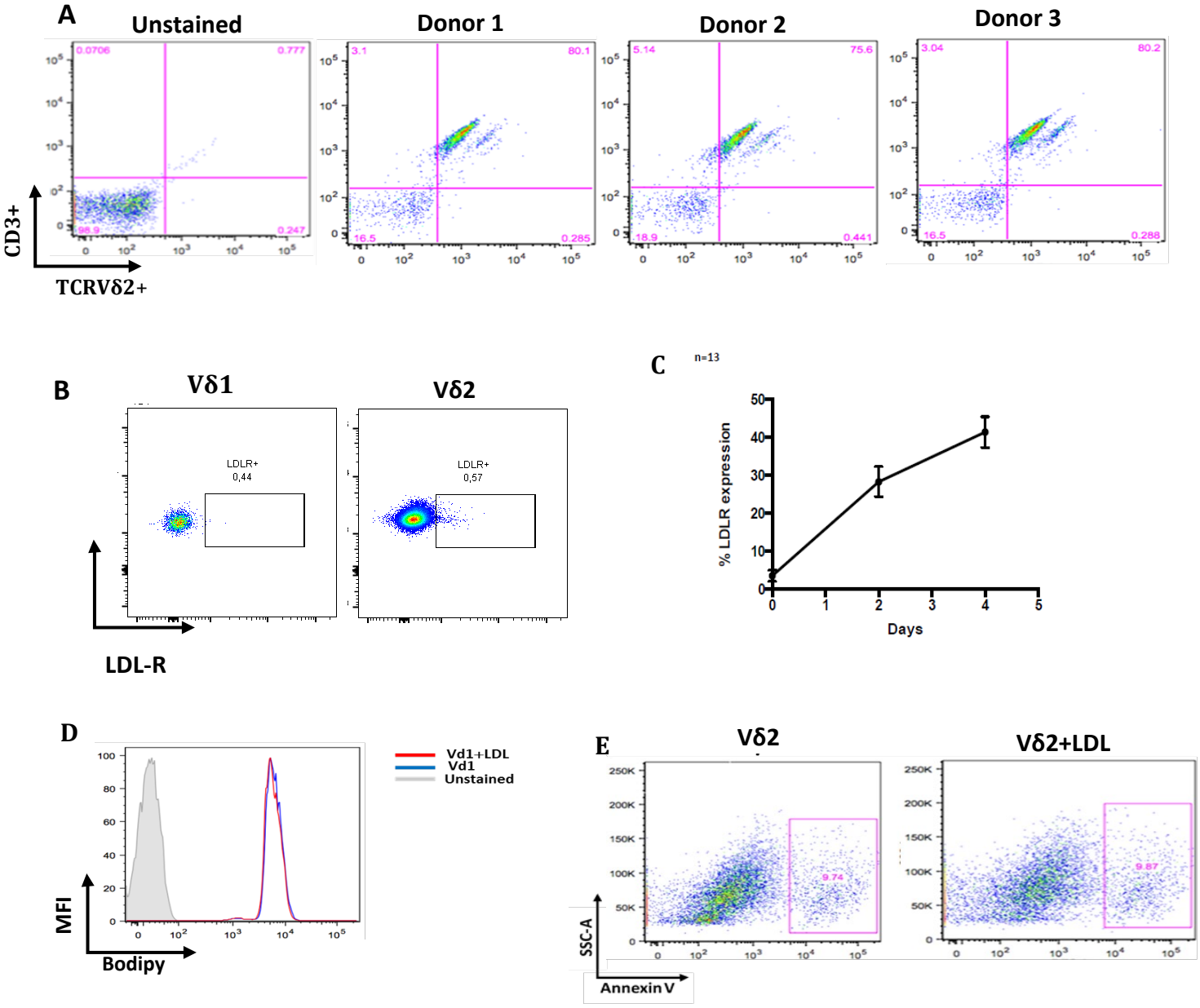


Figure. S1. LDL-cholesterol does not affect the viability of human γδ T cells. (A) Flow cytometry analysis of the percentages of Vδ2⁺ γδ T cells (isolated from different healthy donors) after 14 days in culture with HMB-PP plus IL-2. **(B)** *Ex vivo* LDL-R expression in Vδ1⁺ or Vδ2⁺ γδ T cells. **(C)** Kinetics of LDL-R induction upon Vδ2⁺ γδ T cell activation with HMB-PP plus IL-2. **(D)** Bodipy lipid droplet staining in activated Vδ1⁺ γδ T cells. **(E)** Flow cytometry analysis of Annexin V⁺ (apoptotic) cells in (pre-expanded) Vδ2⁺ γδ T cells upon culture in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours. Data are presented as mean ± s.d. The data represent one of three independent experiments with similar results. *P<0.05,**P<0.01 and ***P<0.001.

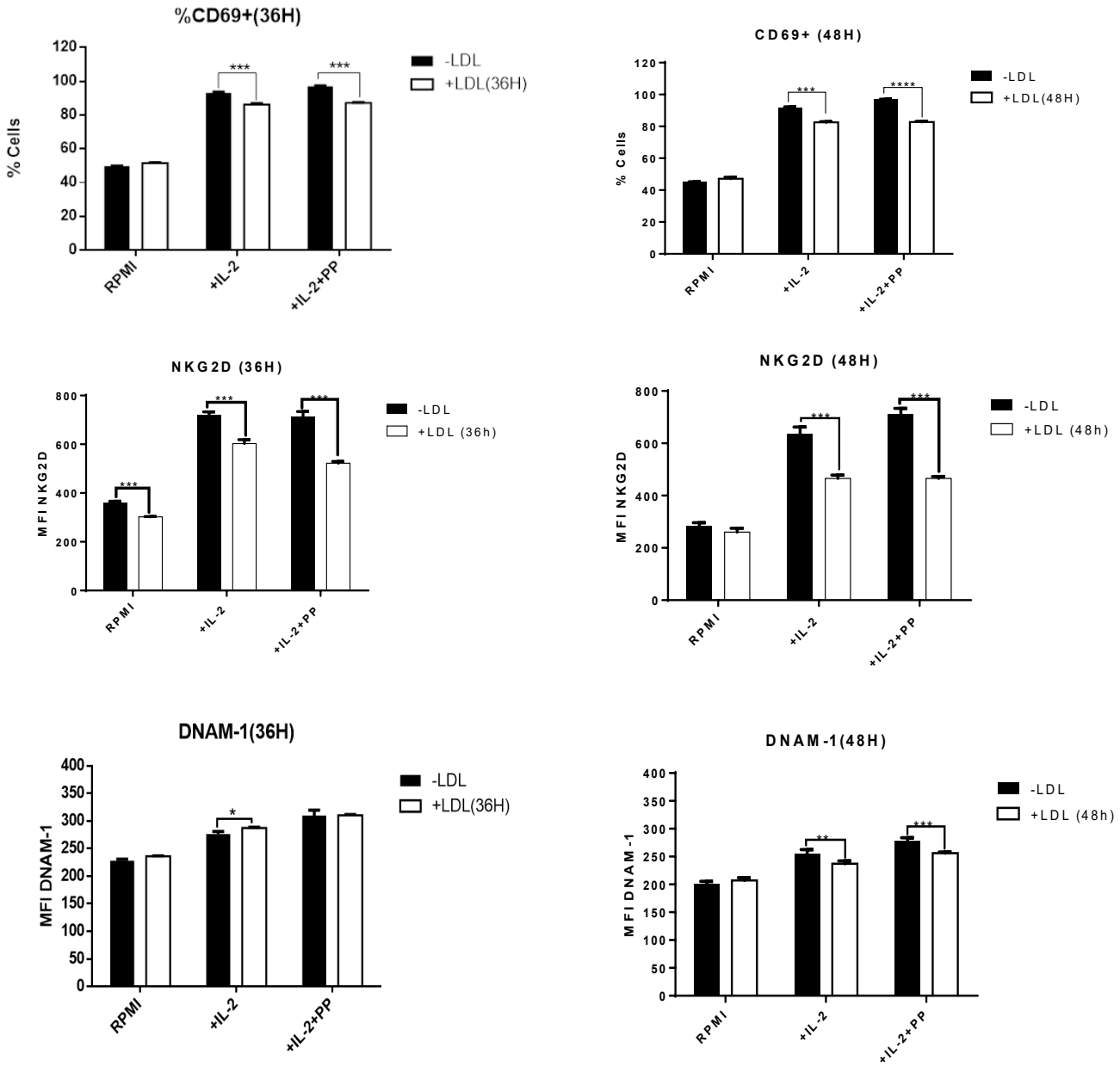


Figure. S2. Effects of LDL-cholesterol on Vδ2$^+$ γδ T cells after 36 or 48 hours of culture (complementary to Figures 2-3). Percentage of positive cells (upper row) or MFI (middle and lower rows) for expression of the indicated markers on Vδ2$^+$ γδ T cells activated for 36 or 48 hours with HMB-PP plus IL-2. Data are presented as mean ± s.d. *P<0.05,**P<0.01 and ***P<0.001.

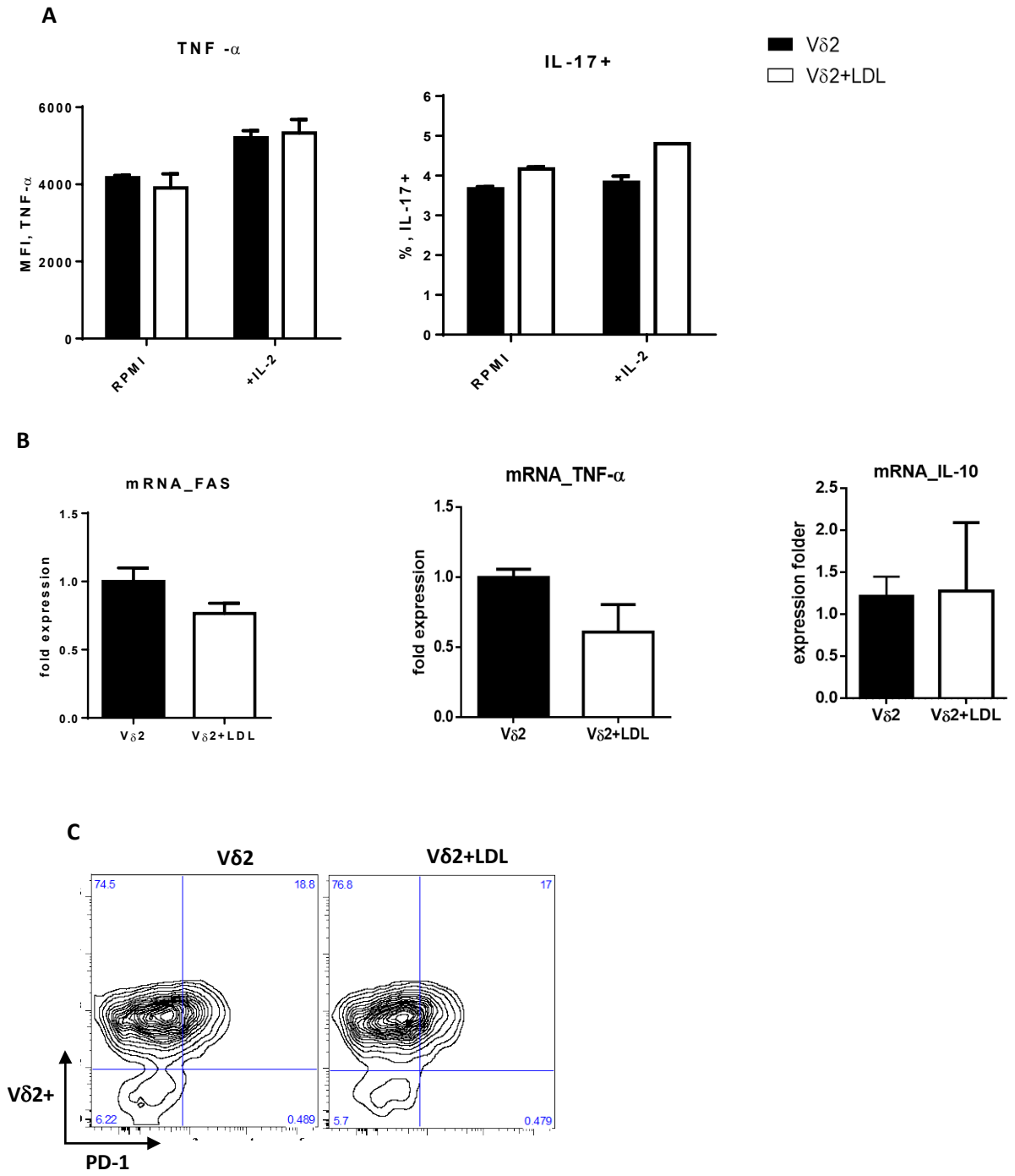


Figure. S3. Effects of LDL-cholesterol on additional immune functions of γδ T cells. γδ T cells (~80% Vδ2⁺) from different donors were cultured in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours and tested for biological function**. (A)** Intracellular cytokine production (TNF-α, left panel and IL-17, right panel) determined by flow cytometry, depicted as MFI (left) or percentage of positive cells (right). **(B)** RT-qPCR analysis of the relative expression of FAS, TNF-α and IL-10. (C) Flow cytometry analysis of PD-1 expression in (pre-expanded) Vδ2+ γδ T cells upon culture in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL) for 72 hours. Data are presented as mean ± s.d. *P<0.05.**P<0.01 and ***P<0.001.

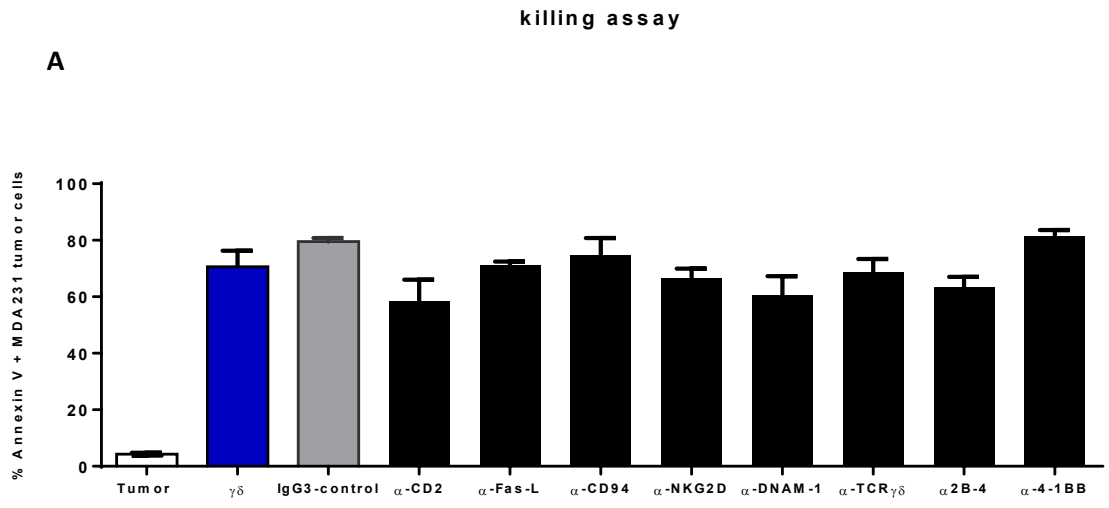


Figure. S4. Effects of receptor blockade on breast cancer cell targeting by γδ T lymphocytes. γδ T cells (~80% Vδ2⁺) were co-cultured for 3 hours with MDA-MB-231 breast cancer cells at 1:10 effector: target in the presence of saturating amounts of the indicated blocking antibodies. The death of target cells (pre-labeled with DDAO-SE dye) was assessed by Annexin-V staining and flow cytometry (A). Data are presented as mean ± s.d. Data are presented as mean ± s.d. *P<0.05,**P<0.01 and ***P<0.001.

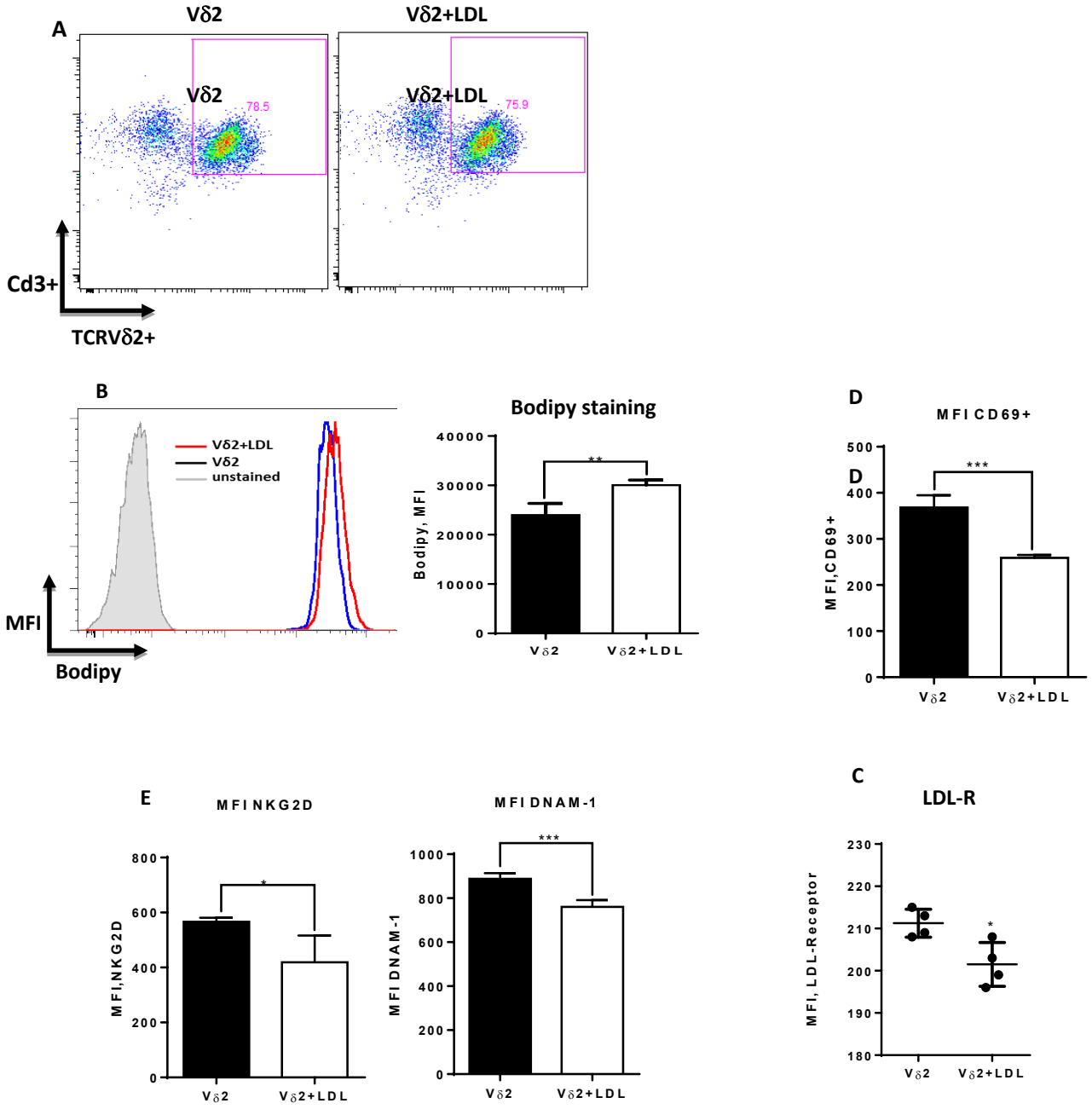


Figure.S5. Pre-expanded Vγ9Vδ2 T cells were cultured in RPMI 1640 medium suplemented with 5% human plasma and 1% of penicillin/streptomycin in the absence (Vδ2) or presence of LDL-cholesterol (Vδ2+LDL), for 72 hours and tested for biological function. (A) Dot plot analysis of percentage of Vγ9Vδ2 T cell populations (B) LDL-cholesterol uptake was assessed by Bodipy. MFI for LDL-R (C), CD69+ (D), NKG2D (left) and DNAM-1 (right) (E) expression on Vδ2+ T cells, cultured in the absence or presence of LDL-cholesterol (Vδ2+LDL). Data are presented as mean ± s.d. Data are presented as mean ± s.d. *P<0.05, **P<0.01 and ***P<0.001.

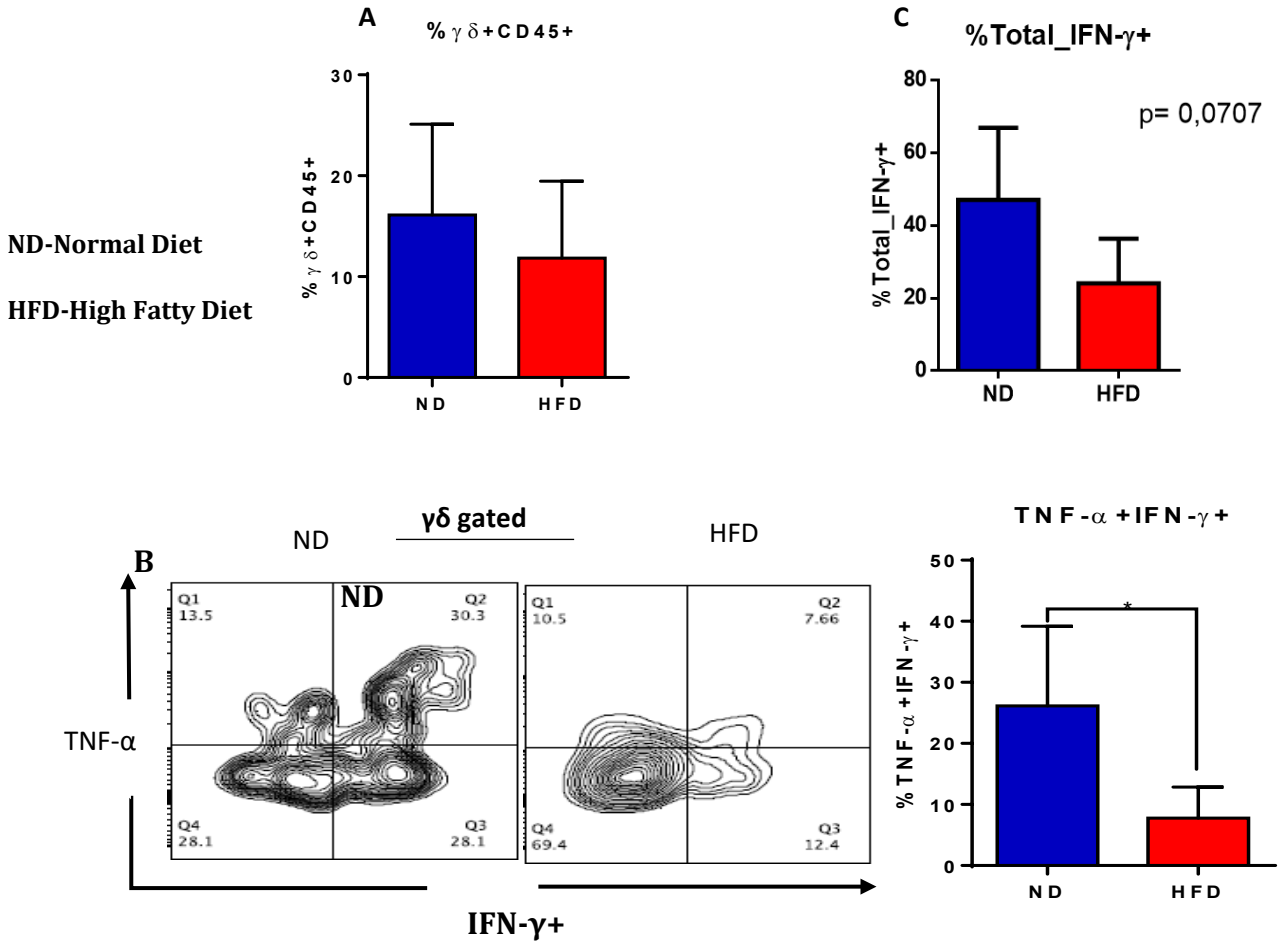


Figure.S6: In vivo High LDL-cholesterol afected TNF-α+IFN-γ+ expression from γδ tumour infiltrating T lymphocytes. C57BL/6 mice were subjected to a high cholesterol diet (HD, 10% fat, 1.25% cholesterol, 0.5% Na cholate diet, Sniff, Germany) and the control group fed the standard (normal) mouse diet (ND). After established diet, (day 9) mice were injected with E0771 murine breast cancer cells into the right axillary mammary fat pad. Then when the presence of tumors was detectable, these animals were sacrificed, and mammary tumors were carefully excised and weighed. Portions of the tumors were analyzed by flow cytometry. (A) Flow cytometry analysis of % of T cells (CD45+γδ+ T cells) and (B) % of TNF-α+IFN-γ+ expression in gated γδ+ T cells. (C) Percentage total of IFN-γ+ prodution. Data are presented as mean ± s.d. Data are presented as mean ± s.d. *P<0.05, **P<0.01 and ***P<0.001.